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U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371

1530.0310002/EKS/EJH

U.S. APPLICATION NO. (IF KNOWN, SEE 37 C.F.R. § 1.5)

To be assigned **09/937604**

INTERNATIONAL APPLICATION NO

PCT/US00/08282

INTERNATIONAL FILING DATE

March 24, 2000

PRIORITY DATE CLAIMED

March 26, 1999

TITLE OF INVENTION

Adjuvant Compositions and Methods for Enhancing Immune Responses to Polynucleotide-Based Vaccines

APPLICANT(S) FOR DO/EO/US

Carl J. Wheeler

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)).
4. ☒ The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
 - a. ☐ is attached hereto (required only if not communicated by the International Bureau).
 - b. ☒ has been communicated by the International Bureau (copy of form PCT/IB/308 attached).
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
7. ☐ Amendments to the claims of the International application under PCT Article 19 (35 U.S.C. 371(c)(3))
 - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
 - b. ☐ have been communicated by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☐ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 372(c)(3)).
9. ☒ An oath or supplemental declaration of the inventor (35 U.S.C. 371(c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:
 - a. Copy of form PCT/IB/308
 - b. Application Data Sheet
 - c. Authorization to Treat a Reply as Incorporating an Extension of Time Under 37 C.F.R. § 1.136(a)(3)

U.S. APPLICATION NO. (if known, see 37 C.F.R. 1.50) 09/937604 To be assigned	INTERNATIONAL APPLICATION NO. PCT/US00/08282	ATTORNEY'S DOCKET NUMBER 1530.0310002/EKS/EJH
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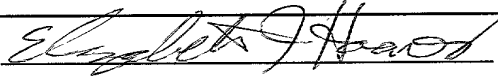
17. <input checked="" type="checkbox"/> The following fees are submitted:	CALCULATIONS PTO USE ONLY																								
Basic National Fee (37 CFR 1.492(a)(1)-(5)): Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO \$1000.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO \$860.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$710.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$690.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) \$ 100.00																									
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<table border="1" style="width:100%; border-collapse: collapse;"> <tr> <th style="width:15%;">Claims</th> <th style="width:20%;">Number Filed</th> <th style="width:15%;">Number Extra</th> <th style="width:15%;">Rate</th> <th style="width:15%;"></th> <th style="width:20%;"></th> </tr> <tr> <td>Total Claims</td> <td>1 - 20 =</td> <td>0</td> <td>X \$18.00</td> <td>\$</td> <td></td> </tr> <tr> <td>Independent Claims</td> <td>1 - 3 =</td> <td>0</td> <td>X \$80.00</td> <td>\$</td> <td></td> </tr> <tr> <td colspan="3">Multiple dependent claim(s) (if applicable)</td> <td>+ \$270.00</td> <td>\$</td> <td></td> </tr> </table>	Claims	Number Filed	Number Extra	Rate			Total Claims	1 - 20 =	0	X \$18.00	\$		Independent Claims	1 - 3 =	0	X \$80.00	\$		Multiple dependent claim(s) (if applicable)			+ \$270.00	\$		
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Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).	\$																								
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Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property	\$																								
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Amount to be refunded:	\$																								
charged:	\$																								

a. ☒ A check in the amount of \$500.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of \$ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 19-0036. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit Under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO: STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C. 1100 New York Avenue, NW, Suite 600 Washington, D.C. 20005-3934	 SIGNATURE <u>Elizabeth J. Haanes, Ph.D.</u> NAME <u>42,613</u> REGISTRATION NUMBER
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09/937604

JC09 Rec'd PCT/PTO 26 SEP 2001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Carl J. Wheeler

Appl. No. *To be Assigned*

Filed: *Herewith*

For: **Adjuvant Compositions and
Methods for Enhancing Immune
Responses to Polynucleotide-
Based Vaccines**

Confirmation No.:

Art Unit: *To Be Assigned*

Examiner: *To Be Assigned*

Atty. Docket: 1530.0310002/EKS/EJH

Preliminary Amendment

Commissioner for Patents
Washington, D.C. 20231

Sir:

In advance of prosecution, please amend the application as follows.

It is not believed that extensions of time or fees for net addition of claims are required beyond those that may otherwise be provided for in documents accompanying this paper. However, if additional extensions of time are necessary to prevent abandonment of this application, then such extensions of time are hereby petitioned under 37 C.F.R. § 1.136(a), and any fees required therefor (including fees for net addition of claims) are hereby authorized to be charged to our Deposit Account No. 19-0036.

In the Specification:

On page 1, below the title and before the first paragraph, please insert the following paragraph:

This Application is the 35 U.S.C. § 371 National Stage of International Application No. PCT/US00/08282, filed March 24, 2000, which was published under PCT Article 21(2) in English, and which claims benefit under 35 U.S.C. § 119(e) to U.S. Provisional Application No. 60/126,340, filed March 26, 1999; and this Application is a Continuation of U.S. Patent Application No. 09/534,943, filed March 24, 2000, which claims benefit under 35 U.S.C. § 119(e) to U.S. Provisional Application No. 60/126,340, filed March 26, 1999. Said International Application No. PCT/US00/08282, U.S. Patent Application No. 09/534,943, and U.S. Provisional Application No. 60/126,340 are incorporated herein by reference in their entireties.

In the Claims:

Please cancel claims 2-66 without prejudice or disclaimer.

09/534,943

Remarks

Upon entry of the foregoing amendment, independent claim 1 is pending in the application. Claims 2-66 are sought to be canceled without prejudice to or disclaimer of the subject matter therein. These changes are believed to introduce no new matter.

Conclusion

It is respectfully believed that this application is now in condition for examination. Early notice to this effect is respectfully requested

The U.S. Patent and Trademark Office is hereby authorized to charge any fee deficiency, or credit any overpayment, to our Deposit Account No. 19-0036.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.



Elizabeth J. Haanes, Ph.D.
Agent for Applicant
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Date: September 26, 2001

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T09260-4092660

Version with markings to show changes made

The following paragraph is inserted on page 1, below the title and before the first paragraph:

This Application is the 35 U.S.C. § 371 National Stage of International Application No. PCT/US00/08282, filed March 24, 2000, which was published under PCT Article 21(2) in English, and which claims benefit under 35 U.S.C. § 119(e) to U.S. Provisional Application No. 60/126,340, filed March 26, 1999; and this Application is a Continuation of U.S. Patent Application No. 09/534,943, filed March 24, 2000, which claims benefit under 35 U.S.C. § 119(e) to U.S. Provisional Application No. 60/126,340, filed March 26, 1999. Said International Application No. PCT/US00/08282, U.S. Patent Application No. 09/534,943, and U.S. Provisional Application No. 60/126,340 are incorporated herein by reference in their entireties.

Claims 2-66 are canceled.

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09/937604

Adjuvant Compositions and Methods for Enhancing Immune Responses to Polynucleotide-Based Vaccines

Field of the Invention

The present invention relates generally to adjuvants, immunogenic compositions, and methods useful for polynucleotide-based vaccination. The present invention provides compositions and methods useful for enhancing immune response, especially the humoral immune response of vertebrates to polynucleotide-based vaccines. In particular, the present invention provides an adjuvant of cytofectin:co-lipid mixture wherein the cytofectin is (\pm)-N-(3-aminopropyl)-N,N-dimethyl-2,3-bis(*syn*-9-tetradeceneyloxy)-1-propanaminium bromide (GAP-DMORIE).

Background of the Invention

In the late 1980s, it was discovered that direct intramuscular (i.m.) injection of lipid-DNA complexes results in measurable protein expression, and that "naked" plasmid DNA (pDNA) is taken up and expressed in muscle to a greater extent than lipid-DNA complexes (Felgner, *Scientific American*, 276(6), 102-106 (1997)).

One of the first applications of pDNA injection technology was the induction of an immune response. In 1991, it was first reported that mice could be immunized against HIV gp120 by i.m. vaccination with gp120 plasmid DNA (Felgner *et al.*, *Nature*, 349, 351-352 (1991)), and that mice could be protected from a lethal challenge of influenza virus after DNA immunization with influenza nucleoprotein (NP) antigen. Protection obtained after immunization with the highly conserved NP antigen extended across 2 different viral strains (Ulmer *et al.*, *Current Opinions In Immunology*, 8, 531-536 (1996)). Numerous publications in the field of polynucleotide-based vaccination followed thereafter (e.g., Boyer *et al.*, *J. Med. Primatology*, 25(3), 242-250 (1996); Boyer *et al.*, *Nature Medicine*, 3(5), 526-532 (1997); Davis *et al.*, *Vaccine*, 15(8), 849-852 (1997); Wang *et al.*, *Vaccine*, 15(8),

821-825 (1997); Agadjanyan *et al.*, *Current Topics In Microbiology And Immunology*, 226, 175-192 (1998); Heppell *et al.*, *Fish & Shellfish Immunology*, 8(4), 271-286 (1998); Lodmell *et al.*, *Nature Medicine*, 4(8), 949-952 (1998); Vanderzanden *et al.*, *Virology*, 246(1), 134-144 (1998)).

5 A major problem frequently encountered in the course of polynucleotide-based vaccination is insufficient or suboptimal humoral response. Often, the antigens or immunogens encoded by the polynucleotide are expressed *in vivo*, but they are not sufficiently immunogenic to raise the antibody titer in the organism to sufficient levels to provide protection against subsequent challenge and/or to maintain the potential for generating therapeutically active antibody levels over extended time periods. To obtain a stronger humoral and/or cellular response, it is common to administer such vaccines in an immunogenic composition containing an adjuvant, a material which enhances the immune response of the patient to the vaccine. Adjuvants are useful generally for improving the immune response of an organism to a particular immunogen and are commonly included in vaccine compositions to increase the amount of antibodies produced and/or to reduce the quantity of immunogen and the frequency of administration.

A variety of adjuvants have been reported to effect differing levels of immune response enhancement to polynucleotide-based vaccination. Examples of such adjuvant materials include semi-synthetic bacterial cell wall-derived mono-phosphoryl lipid A (Sasaki, S., *et al.*, *Infection and Immunity* 65(9), 3250-3258 (1997)), small molecule immunostimulators (Sasaki, S., *et al.*, *Clin. Exp. Immunol.* 111, 30-35 (1998)), and saponins (Sasaki, S., *et al.*, *J. Virology* 72(6), 4391-4939 (1998)). The immune response from i.m. pDNA vaccination has also been enhanced through the use of cationic lipids (Ishii, N., *et al.*, *Aids Res. Hum. Retroviruses* 13(16), 1421-1428 (1997)), Okada, E., *et al.*, *J. Immunology* 159, 3638-3647 (1997); Yokoyama, M., *et al.*, *FEMS Immunol. Med. Microbiol.* 14, 221-230 (1996); Gregoriadis, G., *et al.*, *FEBS Letters* 402, 107-110 (1997); Gramzinski, R.A., *et al.*, *Molecular Medicine* 4, 109-118 (1998); Klavinskis, L.S., *et al.*, *Vaccine* 15(8), 818-820 (1997); Klavinskis, L.S., *et al.*, *J. Immunology* 162, 254-262 (1999); Etchart, N.,

et al., *J. Gen. Virology* 78, 1577-1580 (1997); Norman, J., et al., in Methods in Molecular Medicine, Vol. 9; DNA Vaccines: Methods and Protocols, D.B. Lowrie and R. Whalen, eds., Chapter 16, pp. 185-196 (1999)). Cationic lipids were originally studied as cytofectins to enhance delivery of pDNA into cells *in vitro*; however, further development has led to successful specific applications of protein delivery *in vivo* (Wheeler, C.J., et al., *Proc. Natl. Acad. Sci. USA* 93, 11454-11459 (1996); Stephan, D.J., et al., *Human Gene Therapy* 7, 1803-1812 (1996); DeBruyne, L.A., et al., *Gene Therapy* 5, 1079-1087 (1998)). Accordingly, such cytofectins may be useful for vaccine applications by enhancing delivery of the pDNA into the cells responsible for giving rise to the humoral arm of the immune response, thereby increasing antibody titer levels.

Commonly used adjuvants show low levels of immune response enhancement for vaccination (typically less than 3-fold) and possess undesirable toxicological and manufacturing profiles. In addition, cationic lipids used previously for vaccination show only low levels of humoral enhancement. There is a need for more adjuvant compositions useful for enhancing the immune response of vertebrates to immunization, especially to pDNA vaccination.

Summary of the Invention

The present invention is directed to adjuvant and immunogenic compositions and to methods for the polynucleotide-based vaccination of a vertebrate, to help protect the vertebrate from a disease, to treat a diseased vertebrate, or both. In certain preferred embodiments, the present invention is directed to a method for immunizing a vertebrate by administering to the vertebrate a composition comprising a polynucleotide that encodes for an immunogen, wherein the polynucleotide is complexed with an adjuvant composition comprising GAP-DMORIE. Preferably, the composition may comprise one or more co-lipids. The immunogen-encoding polynucleotide, upon incorporation into the cells of the vertebrate, produces an immunologically effective amount of an immunogen (e.g., an immunogenic protein).

The adjuvant composition of the present invention enhances the immune response of the vertebrate to the immunogen.

One aspect of the present invention is an adjuvant composition comprising a mixture of one or more cytofectins and one or more co-lipids, which adjuvant composition is useful for enhancing the humoral immune response of a vertebrate to an immunogen. Preferably, the adjuvant composition includes the cytofectin GAP-DMORIE and one or more co-lipids. Preferably also, the co-lipid is a neutral lipid such as, for example, a phosphatidylethanolamine. More preferably, the co-lipid is 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-diphytanoyl-*sn*-glycero-3-phosphoethanolamine (DPyPE), and/or 1,2-dimyristoyl-glycer-3-phosphoethanolamine (DMPE). Most preferably, the co-lipid is DPyPE.

Another aspect of the present invention is an immunogenic composition comprising one or more immunogens and an adjuvant composition comprising the cytofectin GAP-DMORIE and one or more co-lipids. In certain embodiments, the source of the immunogen is an immunogen-encoding polynucleotide, such as in the case of a pDNA vaccine. Preferably, in those embodiments, the pDNA or polynucleotide is complexed with an adjuvant composition comprising GAP-DMORIE and one or more co-lipids.

Another aspect of the present invention is a method for immunizing a vertebrate by administering to the vertebrate an immunogenic composition comprising a complex of one or more immunogen-encoding polynucleotides and GAP-DMORIE in an amount sufficient to generate an immune response to the encoded immunogen. Preferably, the immunogenic composition further includes one or more co-lipids such as, for example, DOPE and/or DPyPE. Most preferably, the co-lipid is DPyPE.

The present invention, in contrast to the prior art, is useful for enhancing the humoral immune response of a vertebrate to a polynucleotide-based vaccine, through the use of GAP-DMORIE. Elevation of antibody levels is particularly advantageous in applications where antibody levels from the immunogen-encoding polynucleotide alone are sub-optimal. In a related advantage, if the desired level of antibodies is produced with a given dose of pDNA, the amount of pDNA necessary to reach the

predetermined antibody titer level can be reached using a lower pDNA dose. For pDNA vaccination applications, this advantage is important because acceptable vaccination volumes, coupled with functional limits on the concentration of pDNA, define an upper limit on a given vaccine dose. This advantage is particularly beneficial for vaccines containing multiple plasmids, each of which must be present in sufficient quantity to elicit an immune response to its particular transgene.

Brief Description of the Figures

The foregoing aspects and advantages of the present invention will be readily apparent to one skilled in the art upon reference to the figures and the detailed description which follows.

Figure 1 illustrates the diagrams of Plasmid DNAs. Each vector has a pUC19 origin of replication and a kanamycin resistance gene for plasmid growth in *E. coli* bacteria. CMV = human cytomegalovirus promoter and enhancer; CMV-A = human cytomegalovirus intron A; mRGB = modified rabbit β -globin polyadenylation signal; BGH = bovine growth hormone polyadenylation signal.

Figure 2 illustrates the chemical structures for the cytofectin GAP-DMORIE and the co-lipids DOPE and DPyPE, along with structurally related cytofectins.

Figure 3 is a bar graph demonstrating that the structural elements of cytofectins determine the level of antibody stimulation upon administration. Mice were immunized using pDNA coding for influenza nuclear protein (NP), complexed with various cytofectins (identified on the horizontal axis) formulated as a 1:1 (mol:mol) mixture with DOPE co-lipid. Each animal in the test group (five animals per group) was injected at day "0" and at 3 weeks (boost injection) with 5 μ g pDNA in 50 μ l physiological saline per leg in the rectus femoris muscle, either alone or as a complex with a cytofectin:co-lipid adjuvant. After 6 weeks (3 weeks after the boost), serum was removed from the animals and the NP antibody titers were determined by serial dilution using an ELISA assay. Cytofectin:co-lipid enhancement was evaluated using the ratio of (i) the geometric mean titer (GMT) from a cytofectin-augmented

transfection group to (ii) the GMT from pDNA transfection alone, using an equivalent control group of animals.

Figure 4 is a bar graph illustrating the differential enhancement of anti-NP antibody responses to cytofectins by using DPyPE instead of DOPE as the co-lipid in the adjuvant composition. Mice were immunized and analyzed as described above in connection with Figure 2.

Figure 5 illustrates pDNA-Vaxfectin vaccination dose response and time course. BALB/c mice (8 – 10 weeks old) received bilateral intramuscular injections of 1 µg, 5 µg or 25 µg naked VR4700 plasmid DNA encoding influenza nuclear protein (NP) in 50 µl PBS per muscle (thus, 2 µg, 10 µg or 50 µg total pDNA per time point). A second set of mice received the same pDNA doses formulated with Vaxfectin using a constant pDNA:cationic lipid molar ratio of 4:1. Boost injections were given on days 21 and 42 (arrows). Anti-NP titers were determined from serum samples at 3, 6 and 9 weeks. The lines represent average anti-NP antibody titers + S.E.M. (n = 5 mice per group).

Figure 6A and 6B illustrate Vaxfectin formulation optimization. Control mice received bilateral intramuscular injections of 5 µg naked VR4700 plasmid DNA encoding influenza nuclear protein (NP) in 50 µl PBS per muscle (white bars). The test groups received an equivalent pDNA dose formulated with Vaxfectin at the indicated pDNA:cationic lipid molar ratios (black bars). Boost injections were identical to the initial injections, and were given on day 21. Total NP-specific IgG antibody titers were determined from serum samples on day 42 (3 weeks after the boost). The bars represent average anti-NP titers from two separate experiments (n = 5 – 15 mice per group).

Figure 7A and 7B illustrate the duration of elevated antibody titers induced by Vaxfectin. Mice received bilateral intramuscular injections of either 5 µg naked VR4700 plasmid DNA encoding influenza nuclear protein (NP) in 50 µl PBS per muscle, or the same amount of pDNA formulated with Vaxfectin at a pDNA:cationic lipid molar ratio of 4:1. Identical boost injections were given either on day 21 (A), or on day 21 and again at 3 months (B) (arrows). Total NP-specific IgG antibody titers

were determined from serum samples at various time points. The lines represent average anti-NP titers \pm S.E.M. ($n = 4 - 10$ mice per time point).

Figure 8A, 8B, and 8C illustrate that pDNA formulated with Vaxfectin induces CTL responses that are as robust as those induced with naked pDNA. (A) Mice received bilateral intramuscular injections of 5 μ g VR4700 plasmid DNA encoding influenza nuclear protein (NP) in 50 μ l PBS per muscle on day 0, 21, 42 and 63. A second set of mice received the same pDNA dose formulated with Vaxfectin at the indicated pDNA:cationic lipid molar ratios. (B) Mice received bilateral intramuscular injections of 1 or 25 μ g VR4700 plasmid in 50 μ l PBS per muscle on day 0, 21, 42 and 63. A second set of mice received the same pDNA doses formulated with Vaxfectin at a pDNA:cationic lipid molar ratio of 4:1. (C) Mice received bilateral intramuscular injections of 5 μ g VR4700 plasmid in 50 μ l 150 mM NaP per muscle on day 0 and 21. A second set of mice received the same pDNA dose formulated with Vaxfectin at a pDNA:cationic lipid molar ratio of 4:1. All CTL assays were performed 4-4.5 months after the first injection. The lines represent average specific lysis ($n = 4 - 5$ mice per group).

Figure 9 illustrates the effect of Vaxfectin on β -galactosidase (β -Gal) expression in muscle. Mice received intramuscular injections of 5 μ g naked VR1412 plasmid encoding β -galactosidase. A second group of mice was injected with 5 μ g VR1412 formulated with Vaxfectin at a pDNA:cationic lipid molar ratio of 4:1. At the indicated time points, quadriceps muscles were harvested and assayed for β -Gal activity. The lines represent average reporter gene expression per muscle \pm S.E.M. ($n = 10 - 20$ muscles per group).

Figure 10 illustrates that Vaxfectin enhances humoral immune response in rabbits. Total IgG antibody titers in rabbit serum after i.m. injection of VR4700 plasmid DNA encoding influenza nuclear protein (NP) are shown. New Zealand White rabbits (5 - 6 months old) received a single unilateral injection of either 150 μ g VR4700 plasmid alone or formulated with Vaxfectin (pDNA:cationic lipid = 4:1 molar ratio) in 300 μ l PBS. In one group of animals (triangles), both pDNA and pDNA-Vaxfectin were injected using needle and syringe. In another group of rabbits

(circles), pDNA and pDNA-Vaxfectin were injected using a Biojector needle-free injection device. On day 42 (arrow), rabbits were given an identical boost injection in the contralateral quadriceps muscle. Anti-NP titers were determined from serum samples collected prior to immunization, and at weeks 3, 6, 7, 9, and 13. The lines represent average anti-NP titers + S.E.M. (n = 4 rabbits per group).

Figure 11A, 11B, 11C, 11D, and 11E illustrate that Vaxfectin enhances antigen specific serum antibody responses to 5 different pDNA encoded model antigens. BALB/c mice were immunized with injections of 5 μ g pDNA +/- Vaxfectin into each rectus femoris muscle at 0 and 3 weeks. Data shown are the mean antigen specific IgG titers (+/- SEM) for sera collected 1 day prior to the boost at 3 weeks and at 6 weeks. (n = 20 for all groups, except for NP where n = 29 for Naked NP pDNA; n = 30 for NP pDNA/Vaxfectin and mouse Id where n = 19 for naked pDNA.) A) Anti-influenza NP IgG titers; B) Anti-influenza HEL IgG titers; C) Anti- β -gal IgG titers; D) Anti-Mouse Id IgG titers; E) Anti-Factor IX IgG titers. *Statistically significant difference from titers obtained with naked pDNA, $p \leq 0.05$.

Figure 12A, 12B, 12C, and 12D illustrate that immunization with pDNA formulated with cytofectin induces antigen specific CTL lysis of target cells coated with antigen derived peptides. BALB/c mice were immunized with injections of 5 μ g pDNA +/- Vaxfectin into each rectus femoris muscle at 0 and 3 weeks. Spleens were harvested 11-12 weeks following the initial immunizations and stimulated for 5-6 days with 1 μ M NP₁₄₇₋₁₅₅ or β -gal₈₇₆₋₈₈₄ peptide and 0.5 U/ml of recombinant murine IL-2. Data presented are the average % lysis for 5 spleens in each group. Similar results were obtained in a second assay for both NP and β -gal specific CTL. A) P815 target cells pulsed with NP₁₄₇₋₁₅₅ peptide; B) Unpulsed P815 target cells; C) P815 target cells pulsed with β -gal₈₇₆₋₈₈₄ peptide; D) Unpulsed P815 target cells.

Figure 13A and 13B illustrate the Th1 type isotype profiles of antigen specific antibodies induced with 5 different pDNA encoded model antigens. Serum titers of antigen specific sub-isotypes are presented as a percentage of the sum of IgG1 and IgG2a titers. (n = 20 for all groups, except for NP where n = 29 for Naked NP pDNA; n = 30 for NP pDNA/Vaxfectin and mouse Id where n = 19 for naked pDNA.) A)

Percent of IgG1 and IgG2a at 6 weeks following naked pDNA immunizations. B) Percent of IgG1 and IgG2a at 6 weeks following pDNA/Vaxfectin immunizations.

Figure 14A and 14B illustrate Th1 type cytokine secretion profiles of splenocytes from pDNA/Vaxfectin immunized mice. Spleens were harvested 11-12 weeks following the initial immunizations and were stimulated for 72 hours with 5 μ g/ml of purified NP or β -gal protein. IFN- γ and IL-4 in culture supernatants were determined by ELISA. The data presented are the average concentration of cytokine from cultures of stimulated splenocytes less the concentration of cytokine from cultures of unstimulated splenocytes (+/-SEM). A) Antigen specific IFN- γ response of splenocytes from naked pDNA and pDNA/cytofectin immunized mice (n=10 for each group). B) Antigen specific IL-4 response of splenocytes from naked pDNA and pDNA/Vaxfectin immunized mice (n=10 for each group).

Detailed Description of Preferred Embodiments of the Invention

It will be apparent to one skilled in the art, in view of the following detailed description and the claims appended hereto, that various substitutions and modifications may be made to the present invention without departing from the scope of the invention as claimed.

The present invention is directed to the polynucleotide-based immunization of a vertebrate, to protect from or treat a vertebrate with a disease condition. The present invention includes the use of cytofectin, especially GAP-DMORIE in adjuvants, immunogenic compositions, and methods for immunizing a vertebrate, especially with polynucleotide-based immunogen.

The adjuvant composition of the present invention includes one or more cytofectins and, in preferred embodiments, one or more co-lipids. Cytofectins are cationic lipids. In one embodiment, cytofectin is GAP-DMORIE, which has a structure corresponding to a 2,3-dialkoxy-propanaminium skeleton possessing a unique combination of two linear fourteen-carbon mono-unsaturated alkyl chains and a propylamine substituent on the quaternary nitrogen (See FIG. 2).

GAP-DMORIE contains a set of synergistic structural features, none of which when individually incorporated into the skeleton affords optimal activity. Thus, with reference to FIG. 3, by examining the group DMRIE((±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propanaminium bromide), DLRIE ((±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(dodecyloxy)-1-propanaminium bromide) and DDRIE ((±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(decyloxy)-1-propanaminium bromide), and comparing the group GAP-DMRIE ((±)-N-(3-aminopropyl)-N,N-dimethyl-2,3-(bis-tetradecyloxy)-1-propanaminium bromide), GAP-DLRIE ((±)-N-(3-aminopropyl)-N,N-dimethyl-2,3-(bis-dodecyloxy)-1-propanaminium bromide), and GAP-DPRIE ((±)-N-(3-aminopropyl)-N,N-dimethyl-2,3-(bis-hexadecyloxy)-1-propanaminium bromide), it is evident that fourteen-carbon chains are more active (i.e., elicit greater levels of antibody stimulation) relative to other chain lengths, whether the quaternary nitrogen is substituted with a hydroxyethyl moiety (former group) or with a propylamino moiety (latter group). By comparing DMRIE versus GAP-DMRIE (see FIG. 3), it appears that incorporating a propylamino group *in lieu* of a hydroxyethyl group offers no apparent advantage. Similarly, DMRIE and DMORIE are equally active despite the incorporation of an olefin into the fourteen-carbon chain. However, by incorporating the combination of a propylamino substituent and an olefin moiety, GAP-DMORIE appears to be more active than either DMORIE or GAP-DMRIE, based on the geometric mean titer (GMT) relative to that for pDNA alone (FIG. 3). In addition, DOSPA (2,3-dioleyloxy-N-(2-(sperminecarboxamido)ethyl)-N,N-dimethyl-1-propanaminium pentahydrochloride), which incorporates both an olefin into its eighteen-carbon alkyl chains and an amino-bearing quaternary ammonium substituent, is not only less active than DORIE ((±)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(*syn*-9-octadeceneyloxy)-1-propanaminium bromide), which is equivalent except for quaternary ammonium substitution, but dramatically decreases the level of antibody titers relative to those seen for pDNA alone. The preferred salt of GAP-DMORIE for use in the present invention is the bromide salt; however, all suitable salts of GAP-DMORIE are encompassed by the term "GAP-DMORIE."

For purposes of definition, the term "co-lipid" refers to any hydrophobic material that can be combined with the cytofectin component, *e.g.*, GAP-DMORIE. The co-lipid of the present invention can be amphipathic lipids and neutral lipids. Amphipathic lipids include phospholipids, *e.g.*, phosphatidylethanolamines and phosphatidylcholines. Neutral lipids include cholesterol. In one preferred embodiment, phosphatidylethanolamines include DOPE, DMPE, and DPyPE. DOPE and DpyPE are particularly preferred; the most preferred co-lipid is DpyPE, which comprises two phytanoyl substituents incorporated into the diacylphosphatidylethanolamine skeleton. As illustrated by FIG. 3, the combination of the cytofectin GAP-DMORIE with the co-lipid DPyPE results in a synergistic effect to further enhance the humoral immune response, as evidenced by the level of antibody titers from pDNA immunization.

According to the present invention, cytofectins and co-lipids may be mixed or combined in a number of ways to produce a variety of adjuvant compositions of non-covalently bonded macroscopic structures, *e.g.*, liposomes, multilamellar vesicles, unilamellar vesicles, micelles, and simple films. The cytofectins and co-lipids can be mixed in a variety of molar ratios. Preferably, the molar ratio of GAP-DMORIE and co-lipid is from about 9:1 to about 1:9, more preferably, the molar ratio is from about 4:1 to about 1:4, or from about 2:1 to about 1:2. Most preferably, the molar ratio is about 1:1.

The cytofectins and co-lipids can be dissolved in a solvent to increase homogeneity of the mixture. Suitable solvents include chloroform. For example, GAP-DMORIE can be mixed with one or more co-lipids in chloroform, the mixture is subsequently evaporated under vacuum to form a dried thin layer of film on the inner surface of a glass vessel, *e.g.*, a Rotovap round-bottomed flask. Such dried mixture can be suspended in an aqueous solvent where the amphipathic lipid component molecules self-assemble into homogenous lipid vesicles. These lipid vesicles can subsequently be processed by any methods used in the art to have a selected mean diameter of uniform size prior to complexing with other entities, *e.g.*, pDNA. The sonication of a lipid solution is described in Felgner *et al.*, *Proc. Natl. Acad. Sci. USA*

84,7413-7417 (1987) and in U.S. Pat. No. 5,264,618, the disclosure of which is incorporated herein by reference.

The adjuvant compositions of the present invention may include additives such as hydrophobic and amphiphilic additives. For example, the adjuvant composition can include sterols, fatty acids, gangliosides, glycolipids, lipopeptides, liposaccharides, neobees, niosomes, prostaglandins or sphingolipids. The amount of additives included in the adjuvant may be any including from about 0.1 mol % to about 99.9 mol %, from about 1 mol% to about 50 mol %, and from about 2 mol% to about 25 mol%, relative to total amount of lipid. These additives can also be included in an immunogenic composition containing the adjuvant composition of the present invention.

The immunogenic composition of the present invention includes an adjuvant composition as described above and an immunogen. An "immunogen" is meant to encompass any antigenic or immunogenic polypeptides including poly-aminoacid materials having epitopes or combinations of epitopes, and immunogen-encoding polynucleotides. In addition, an "immunogen" is also meant to encompass any polysaccharide material useful in generating immune response. As used herein, an antigenic polypeptide or an immunogenic polypeptide is a polypeptide which, when introduced into a vertebrate, reacts with the immune system molecules of the vertebrate, i.e., is antigenic, and/or induces an immune response in the vertebrate, i.e., is immunogenic. It is quite likely that an immunogenic polypeptide will also be antigenic, but an antigenic polypeptide, because of its size or conformation, may not necessarily be immunogenic. Examples of antigenic and immunogenic polypeptides include, but are not limited to, polypeptides from infectious agents such as bacteria, viruses, parasites, or fungi, allergens such as those from pet dander, plants, dust, and other environmental sources, as well as certain self polypeptides, for example, tumor-associated antigens.

Antigenic and immunogenic polypeptides of the present invention can be used to prevent or treat, i.e., cure, ameliorate, lessen the severity of, or prevent or reduce

contagion of viral, bacterial, fungal, and parasitic infectious diseases, as well as to treat allergies.

In addition, antigenic and immunogenic polypeptides of the present invention can be used to prevent or treat, i.e., cure, ameliorate, or lessen the severity of cancer including, but not limited to, cancers of oral cavity and pharynx (i.e., tongue, mouth, pharynx), digestive system (i.e., esophagus, stomach, small intestine, colon, rectum, anus, anal canal, anorectum, liver, gallbladder, pancreas), respiratory system (i.e., larynx, lung), bones, joints, soft tissues (including heart), skin, melanoma, breast, reproductive organs (i.e., cervix, endometrium, ovary, vulva, vagina, prostate, testis, penis), urinary system (i.e., urinary bladder, kidney, ureter, and other urinary organs), eye, brain, endocrine system (i.e., thyroid and other endocrine), lymphoma (i.e., hodgkin's disease, non-hodgkin's lymphoma), multiple myeloma, leukemia (i.e., acute lymphocytic leukemia, chronic lymphocytic leukemia, acute myeloid leukemia, chronic myeloid leukemia).

Examples of viral antigenic and immunogenic polypeptides include, but are not limited to, adenovirus polypeptides, alphavirus polypeptides, calicivirus polypeptides, e.g., a calicivirus capsid antigen, coronavirus polypeptides, distemper virus polypeptides, Ebola virus polypeptides, enterovirus polypeptides, flavivirus polypeptides, hepatitis virus (AE) polypeptides, e.g., a hepatitis B core or surface antigen, herpesvirus polypeptides, e.g., a herpes simplex virus or varicella zoster virus glycoprotein, immunodeficiency virus polypeptides, e.g., the human immunodeficiency virus envelope or protease, infectious peritonitis virus polypeptides, influenza virus polypeptides, e.g., an influenza A hemagglutinin, neuraminidase, or nucleoprotein, leukemia virus polypeptides, Marburg virus polypeptides, orthomyxovirus polypeptides, papilloma virus polypeptides, parainfluenza virus polypeptides, e.g., the hemagglutinin/neuraminidase, paramyxovirus polypeptides, parvovirus polypeptides, pestivirus polypeptides, picorna virus polypeptides, e.g., a poliovirus capsid polypeptide, pox virus polypeptides, e.g., a vaccinia virus polypeptide, rabies virus polypeptides, e.g., a

rabies virus glycoprotein G, reovirus polypeptides, retrovirus polypeptides, and rotavirus polypeptides.

Examples of bacterial antigenic and immunogenic polypeptides include, but are not limited to, Actinomyces polypeptides, Bacillus polypeptides, Bacteroides polypeptides, Bordetella polypeptides, Bartonella polypeptides, Borrelia polypeptides, e.g., B. burgdorferi OspA, Brucella polypeptides, Campylobacter polypeptides, Capnocytophaga polypeptides, Chlamydia polypeptides, Clostridium polypeptides, Corynebacterium polypeptides, Coxiella polypeptides, Dermatophilus polypeptides, Enterococcus polypeptides, Ehrlichia polypeptides, Escherichia polypeptides, Francisella polypeptides, Fusobacterium polypeptides, Haemobartonella polypeptides, Haemophilus polypeptides, e.g., H. influenzae type b outer membrane protein, Helicobacter polypeptides, Klebsiella polypeptides, L-form bacteria polypeptides, Leptospira polypeptides, Listeria polypeptides, Mycobacteria polypeptides, Mycoplasma polypeptides, Neisseria polypeptides, Neorickettsia polypeptides, Nocardia polypeptides, Pasteurella polypeptides, Peptococcus polypeptides, Peptostreptococcus polypeptides, Pneumococcus polypeptides, Proteus polypeptides, Pseudomonas polypeptides, Rickettsia polypeptides, Rochalimaea polypeptides, Salmonella polypeptides, Shigella polypeptides, Staphylococcus polypeptides, Streptococcus polypeptides, e.g., S. pyogenes M proteins, Treponema polypeptides, and Yersinia polypeptides, e.g., Y. pestis F1 and V antigens.

Examples of fungal immunogenic and antigenic polypeptides include, but are not limited to, Absidia polypeptides, Acremonium polypeptides, Alternaria polypeptides, Aspergillus polypeptides, Basidiobolus polypeptides, Bipolaris polypeptides, Blastomyces polypeptides, Candida polypeptides, Coccidioides polypeptides, Conidiobolus polypeptides, Cryptococcus polypeptides, Curvalaria polypeptides, Epidermophyton polypeptides, Exophiala polypeptides, Geotrichum polypeptides, Histoplasma polypeptides, Madurella polypeptides, Malassezia polypeptides, Microsporum polypeptides, Moniliella polypeptides, Mortierella polypeptides, Mucor polypeptides, Paecilomyces polypeptides, Penicillium polypeptides, Phialemonium polypeptides, Phialophora polypeptides, Prototheca

polypeptides, Pseudallescheria polypeptides, Pseudomicrodochium polypeptides, Pythium polypeptides, Rhinosporidium polypeptides, Rhizopus polypeptides, Scolecobasidium polypeptides, Sporothrix polypeptides, Stemphylium polypeptides, Trichophyton polypeptides, Trichosporon polypeptides, and Xylohypha polypeptides.

5 Examples of protozoan parasite immunogenic and antigenic polypeptides include, but are not limited to, Babesia polypeptides, Balantidium polypeptides, Besnoitia polypeptides, Cryptosporidium polypeptides, Eimeria polypeptides, Encephalitozoon polypeptides, Entamoeba polypeptides, Giardia polypeptides, Hammondia polypeptides, Hepatozoon polypeptides, Isospora polypeptides, 10 Leishmania polypeptides, Microsporidia polypeptides, Neospora polypeptides, Nosema polypeptides, Pentatrichomonas polypeptides, Plasmodium polypeptides, e.g., *P. falciparum* circumsporozoite (PfCSP), sporozoite surface protein 2 (PfSSP2), carboxyl terminus of liver stage antigen 1 (PfLSA1 c-term), and exported protein 1 (PfExp-1), Pneumocystis polypeptides, Sarcocystis polypeptides, Schistosoma 15 polypeptides, Theileria polypeptides, Toxoplasma polypeptides, and Trypanosoma polypeptides.

 Examples of helminth parasite immunogenic and antigenic polypeptides include, but are not limited to, Acanthocheilonema polypeptides, Aelurostrongylus polypeptides, Ancylostoma polypeptides, Angiostrongylus polypeptides, Ascaris 20 polypeptides, Brugia polypeptides, Bunostomum polypeptides, Capillaria polypeptides, Chabertia polypeptides, Cooperia polypeptides, Crenosoma polypeptides, Dictyocaulus polypeptides, Dioctophyme polypeptides, Dipetalonema polypeptides, Diphyllbothrium polypeptides, Diplydium polypeptides, Dirofilaria polypeptides, Dracunculus polypeptides, Enterobius polypeptides, Filaroides 25 polypeptides, Haemonchus polypeptides, Lagochilascaris polypeptides, Loa polypeptides, Mansonella polypeptides, Muellerius polypeptides, Nanophyetus polypeptides, Necator polypeptides, Nematodirus polypeptides, Oesophagostomum polypeptides, Onchocerca polypeptides, Opisthorchis polypeptides, Ostertagia polypeptides, Parafilaria polypeptides, Paragonimus polypeptides, Parascaris 30 polypeptides, Physaloptera polypeptides, Protostrongylus polypeptides, Setaria

polypeptides, Spirocerca polypeptides Spirometra polypeptides, Stephanofilaria polypeptides, Strongyloides polypeptides, Strongylus polypeptides, Thelazia polypeptides, Toxascaris polypeptides, Toxocara polypeptides, Trichinella polypeptides, Trichostrongylus polypeptides, Trichuris polypeptides, Uncinaria polypeptides, and Wuchereria polypeptides.

Examples of ectoparasite immunogenic and antigenic polypeptides include, but are not limited to, polypeptides (including protective antigens as well as allergens) from fleas; ticks, including hard ticks and soft ticks; flies, such as midges, mosquitos, sand flies, black flies, horse flies, horn flies, deer flies, tsetse flies, stable flies, myiasis-causing flies and biting gnats; ants; spiders, lice; mites; and true bugs, such as bed bugs and kissing bugs.

Examples of tumor-associated antigenic and immunogenic polypeptides include, but are not limited to, tumor-specific immunoglobulin variable regions, GM2, Tn, sTn, Thompson-Friedenreich antigen (TF), Globo H, Le(y), MUC1, MUC2, MUC3, MUC4, MUC5AC, MUC5B, MUC7, carcinoembryonic antigens, beta chain of human chorionic gonadotropin (hCG beta), HER2/neu, PSMA, EGFRvIII, KSA, PSA, PSCA, GP100, MAGE 1, MAGE 2, TRP 1, TRP 2, tyrosinase, MART-1, PAP, CEA, BAGE, MAGE, RAGE, and related proteins.

Also included as polypeptides of the present invention are fragments or variants of the foregoing polypeptides, and any combination of the foregoing polypeptides. Additional polypeptides may be found, for example in "Foundations in Microbiology," Talaro, et al., eds., McGraw-Hill Companies (Oct., 1998), Fields, et al., "Virology," 3d ed., Lippincott-Raven (1996), "Biochemistry and Molecular Biology of Parasites," Marr, et al., eds., Academic Press (1995), and Deacon, J., "Modern Mycology," Blackwell Science Inc (1997), which are incorporated herein by reference.

The immunogen-encoding polynucleotide is intended to encompass a singular "polynucleotide" as well as plural "polynucleotides," and refers to an isolated molecule or construct. The immunogen-encoding polynucleotides include nucleotide sequences, nucleic acids, nucleic acid oligomers, messenger RNA (mRNA), DNA

(e.g., pDNAs, derivatives of pDNA, linear DNA), or fragments of any of thereof. The immunogen-encoding polynucleotides may be provided in linear, circular, e.g., plasmid, or branched form as well as double-stranded or single-stranded form. The immunogen-encoding polynucleotides may comprise a conventional phosphodiester bond or a non-conventional bond, e.g., an amide bond, such as found in peptide nucleic acids (PNA).

According to the present invention, the immunogen-encoding polynucleotide can be part of a circular or linearized plasmid containing a non-infectious and non-integrating polynucleotide. A non-infectious polynucleotide is a polynucleotide that does not infect vertebrate cells while a non-integrating polynucleotide does not integrate into the genome of vertebrate cells. A linearized plasmid is a plasmid that was previously circular but has been linearized, for example, by digestion with a restriction endonuclease. The immunogen-encoding polynucleotide may comprise a sequence that directs the secretion of a polypeptide.

The form of immunogen-encoding polynucleotides depends in part on the desired kinetics and duration of expression. When long-term delivery of a protein encoded by a polynucleotide is desired, the preferred form is DNA. Alternatively, when short-term transgene protein delivery is desired, the preferred form is mRNA, since mRNA can be rapidly translated into polypeptide, however RNA may be degraded more quickly than DNA.

In one embodiment, the immunogen-encoding polynucleotide is RNA, e.g., messenger RNA (mRNA). Methods for introducing RNA sequences into mammalian cells is described in U.S. Patent No. 5,580,859, the disclosure of which is incorporated herein by reference. A viral alphavector, a non-infectious vector useful for administering RNA, may be used to introduce RNA into mammalian cells. Methods for the *in vivo* introduction of alphaviral vectors to mammalian tissues are described in Altman-Hamamdzcic, S., *et al.*, *Gene Therapy* 4, 815-822 (1997), the disclosure of which is incorporated herein by reference.

Preferably, the immunogen-encoding polynucleotide is DNA. In the case of DNA, a promoter is preferably operably linked to the nucleotide sequence encoding

for the immunogen. The promoter can be a cell-specific promoter that directs substantial transcription of the DNA only in predetermined cells. Other transcription control elements, besides a promoter, can be included with the polynucleotide to direct cell-specific transcription of the DNA. An operable linkage is a linkage in which a polynucleotide encoding for an immunogenic molecule is connected to one or more regulatory sequences in such a way as to place expression of the immunogen under the influence or control of the regulatory sequence(s). Two DNA sequences (such as a coding sequence and a promoter region sequence linked to the 5' end of the coding sequence) are operably linked if induction of promoter function results in the transcription of mRNA encoding for the desired immunogen and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the expression regulatory sequences to direct the expression of the immunogen, or (3) interfere with the ability of the DNA template to be transcribed. Thus, a promoter region would be operably linked to a DNA sequence if the promoter was capable of effecting transcription of that DNA sequence.

The immunogen-encoding polynucleotide, *e.g.*, pDNA, mRNA, polynucleotide or nucleic acid oligomer can be solubilized in any of various buffers prior to mixing or complexing with the adjuvant components, *e.g.*, cytofectins and co-lipids. Suitable buffers include phosphate buffered saline (PBS), normal saline, Tris buffer, and sodium phosphate. Insoluble polynucleotides can be solubilized in a weak acid or weak base, and then diluted to the desired volume with a buffer. The pH of the buffer may be adjusted as appropriate. In addition, a pharmaceutically acceptable additive can be used to provide an appropriate osmolarity. Such additives are within the purview of one skilled in the art.

According to the present invention, the immunogen-encoding polynucleotides can be complexed with the adjuvant compositions of the present invention by any means known in the art, *e.g.*, by mixing a pDNA solution and a solution of cytofectin/co-lipid liposomes. In one embodiment, the concentration of each of the constituent solutions is adjusted prior to mixing such that the desired final

pDNA/cytfectin:co-lipid ratio and the desired pDNA final concentration will be obtained upon mixing the two solutions. For example, if the desired final solution is to be physiological saline (0.9% weight/volume), both pDNA and cytofectin:co-lipid liposomes are prepared in 0.9% saline and then simply mixed to produce the desired complex. The cytofectin:co-lipid liposomes can be prepared by any means known in the art. For example, one can hydrate a thin film of GAP-DMORIE and co-lipid mixture in an appropriate volume of aqueous solvent by vortex mixing at ambient temperatures for about 1 minute. Preparation of a thin film of cytofectin and co-lipid mixture is known to a skilled artisan and can be prepared by any suitable techniques. For example, one can mix chloroform solutions of the individual components to generate an equimolar solute ratio and subsequently aliquot a desired volume of the solutions into a suitable container where the solvent can be removed by evaporation, *e.g.*, first with a stream of dry, inert gas such as argon and then by high vacuum treatment.

According to the present invention, the immunogenic composition of the present invention can be used to immunize a vertebrate. The term "vertebrate" is intended to encompass a singular "vertebrate" as well as plural "vertebrates", and comprises mammalian and avian species, as well as fish. The method for immunizing a vertebrate includes administering to the vertebrate an immunogenic composition of the present invention in an amount sufficient to generate an immune response to the immunogen.

The immunogenic compositions of the present invention may be administered according to any of various methods known in the art. For example, U.S. Patent No. 5,676,954 reports on the injection of genetic material, complexed with cationic lipid carriers, into mice. Also, U.S. Patent Nos. 5,589,466, 5,693,622, 5,580,859, 5,703,055, and PCT international patent application PCT/US94/06069 (WO 94/29469), the disclosures of which are incorporated herein by reference, provide methods for delivering DNA-cationic lipid complexes to mammals.

Specifically, the immunogenic compositions of the present invention may be administered to any tissue of a vertebrate, including, but not limited to, muscle, skin,

brain, lung, liver, spleen, bone marrow, thymus, heart, lymph, blood, bone, cartilage, mucosal tissue, pancreas, kidney, gall bladder, stomach, intestine, testis, ovary, uterus, vaginal tissue, rectum, nervous system, eye, gland, tongue and connective tissue.

Preferably, the compositions are administered to skeletal muscle. The immunogenic compositions of the invention may also be administered to a body cavity, including, but not limited to, the lung, mouth, nasal cavity, stomach, peritoneum, intestine, heart chamber, vein, artery, capillary, lymphatic, uterus, vagina, rectum, and ocular cavity.

Preferably, the immunogenic compositions of the present invention are administered by intramuscular (i.m.) or subcutaneous (s.c.) routes. Other suitable routes of administration include transdermal, intranasal, inhalation, intratracheal, transmucosal (*i.e.*, across a mucous membrane), intra-cavity (*e.g.*, oral, vaginal, or rectal), intraocular, vaginal, rectal, intraperitoneal, intrainestinal and intravenous (i.v.) administration.

Any mode of administration can be used so long as the administration results in desired immune response. Administration means of the present invention include, but not limited to, needle injection, catheter infusion, biolistic injectors, particle accelerators (*i.e.*, "gene guns" or pneumatic "needleless" injectors -- for example, Med-E-Jet (Vahlsing, H., *et al.*, *J. Immunol. Methods* 171,11-22 (1994)), Pigjet (Schrijver, R., *et al.*, *Vaccine* 15, 1908-1916 (1997)), Biojector (Davis, H., *et al.*, *Vaccine* 12, 1503-1509 (1994); Gramzinski, R., *et al.*, *Mol. Med.* 4, 109-118 (1998)), AdvantaJet, Medijector, gelfoam sponge depots, other commercially available depot materials (*e.g.*, hydrogels), osmotic pumps (*e.g.*, Alza minipumps), oral or suppositorial solid (tablet or pill) pharmaceutical formulations, topical skin creams, and decanting, use of polynucleotide coated suture (Qin *et al.*, *Life Sciences* 65, 2193-2203 (1999)) or topical applications during surgery. The preferred modes of administration are intramuscular needle-based injection and intranasal application as an aqueous solution.

Determining an effective amount of an immunogenic composition depends upon a number of factors including, for example, the chemical structure and biological activity of the substance, the age and weight of the subject, and the route of

administration. The precise amount, number of doses, and timing of doses can be readily determined by those skilled in the art.

In certain embodiments, the immunogenic composition is administered as a pharmaceutical composition. Such a pharmaceutical composition can be formulated according to known methods, whereby the substance to be delivered is combined with a pharmaceutically acceptable carrier vehicle. Suitable vehicles and their preparation are described, for example, in Remington's Pharmaceutical Sciences, 16th Edition, A. Osol, ed., Mack Publishing Co., Easton, PA (1980), and Remington's Pharmaceutical Sciences, 19th Edition, A.R. Gennaro, ed., Mack Publishing Co., Easton, PA (1995).

The pharmaceutical composition can be formulated as an emulsion, gel, solution, suspension, lyophilized form, or any other form known in the art. In addition, the pharmaceutical composition can also contain pharmaceutically acceptable additives including, for example, diluents, binders, stabilizers, and preservatives.

Administration of pharmaceutically acceptable salts of the polynucleotide constructs described herein is preferred. Such salts can be prepared from pharmaceutically acceptable non-toxic bases including organic bases and inorganic bases. Salts derived from inorganic bases include sodium, potassium, lithium, ammonium, calcium, magnesium, and the like. Salts derived from pharmaceutically acceptable organic non-toxic bases include salts of primary, secondary, and tertiary amines, basic amino acids, and the like.

For aqueous pharmaceutical compositions used *in vivo*, use of sterile pyrogen-free water is preferred. Such formulations will contain an effective amount of the immunogenic composition together with a suitable amount of vehicle in order to prepare pharmaceutically acceptable compositions suitable for administration to a vertebrate.

The present invention also provides kits for use in delivering a polypeptide to a vertebrate. Each kit includes a container holding 1 ng to 30 mg of an immunogen-encoding polynucleotide which operably encodes an immunogen within vertebrate cells *in vivo*. Furthermore, each kit includes, in the same or in a different container, an adjuvant composition comprising GAP-DMORIE and a co-lipid. Any of

components of the pharmaceutical kits can be provided in a single container or in multiple containers. Preferably, the kit includes from about 1 ng to about 30 mg of a immunogen-encoding polynucleotide, more preferably, the kit includes from about 100 ng to about 10 mg of a immunogen-encoding polynucleotide.

5 Any suitable container or containers may be used with pharmaceutical kits. Examples of containers include, but are not limited to, glass containers, plastic containers, or strips of plastic or paper.

Each of the pharmaceutical kits may further comprise an administration means. Means for administration include, but are not limited to syringes and needles, catheters, biolistic injectors, particle accelerators, *i.e.*, "gene guns," pneumatic "needleless" injectors, gelfoam sponge depots, other commercially available depot materials, *e.g.*, hydrogels, osmotic pumps, and decanting or topical applications during surgery. Each of the pharmaceutical kits may further comprise sutures, *e.g.*, coated with the immunogenic composition (Qin et al., *Life Sciences* (1999) 65:2193-2203).

15 The kit can further comprise an instruction sheet for administration of the composition to a vertebrate. The polynucleotide components of the pharmaceutical composition are preferably provided as a liquid solution or they may be provided in lyophilized form as a dried powder or a cake. If the polynucleotide is provided in lyophilized form, the dried powder or cake may also include any salts, entry enhancing agents, transfection facilitating agents, and additives of the pharmaceutical composition in dried form. Such a kit may further comprise a container with an exact amount of sterile pyrogen-free water, for precise reconstitution of the lyophilized components of the pharmaceutical composition.

25 The container in which the pharmaceutical composition is packaged prior to use can comprise a hermetically sealed container enclosing an amount of the lyophilized formulation or a solution containing the formulation suitable for a pharmaceutically effective dose thereof, or multiples of an effective dose. The pharmaceutical composition is packaged in a sterile container, and the hermetically sealed container is designed to preserve sterility of the pharmaceutical formulation

until use. Optionally, the container can be associated with administration means and/or instruction for use.

The following examples are included for purposes of illustration only and are not intended to limit the scope of the present invention, which is defined by the
5 appended claims.

Examples

The following examples demonstrate the surprising finding that various GAP-DMORIE:co-lipid complexed with an antigen-encoding pDNA can enhance
10 subsequent immune response compared to presently known nucleic acid immunization methods when administered into murine or rabbit tissues.

Materials and Methods

The following materials and methods apply generally to all the examples
15 disclosed herein. Specific materials and methods are disclosed in each example, as necessary.

Reagents

Sterile USP water and saline solutions were purchased from Baxter (Deerfield,
20 IL). All other chemicals and solvents were purchased either from Sigma Chem. Corp. (St. Louis, MO) or Gallade Chemical (Escondido, CA). Both the 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and 1,2-diphytanoyl-*sn*-glycero-3-phosphoethanolamine (DPyPE) were purchased as chloroform solutions from Avanti Polar Lipids, Inc. (Alabaster, Alabama).

Preparation of adjuvant and immunogenic compositions

(±)-N-(3-aminopropyl)-N,N-dimethyl-2,3-bis(*syn*-9-tetradeceneyloxy)-1-propanaminium bromide (GAP-DMORIE, also called VC 1052) was synthesized using the published procedure for preparing the analogue cytofectin GAP-DLRIE
30 (Wheeler *et al.*, *Proc. Natl. Acad. Sci.* 93, 11454-11459 (1996)). Specifically,

substituting *syn*-9-tetradecenyl methane sulfonate for dodecenyl methane sulfonate in the initial *bis*-alkylation of 3-dimethylamino-1,2-propanediol yielded the desired dialkenyl amine. Quatranization with 3-bromopropylphthalimide, followed by deprotection of the protected primary amine with hydrazine and extractive purification and sub-micron filtration afforded pure GAP-DMORIE as judged by analytical thin layer chromatography. Product identity was confirmed using high resolution proton NMR and infrared (IR) spectroscopies.

Cytofectin:co-lipid mixtures were prepared using the rehydrated thin-film method. Briefly, dried films were prepared in 2 ml sterile glass vials by evaporating the chloroform under a stream of nitrogen, and placing the vials under vacuum overnight to remove solvent traces. Each vial contained 1.5 μ mole each of a cytofectin and a co-lipid. Liposomes were prepared by adding 1 ml SWFI (sterile water for injection, VWR, Philadelphia, PA) per vial followed by continuous vortexing for 5 min on the highest setting of a Genie Vortex Mixer (Fisher Scientific, Pittsburgh, PA). The resulting liposome solution contained 1.5 mM cytofectin. Formulations were prepared at final pDNA(phosphate):cationic lipid molar ratios of 8:1, 4:1, and 2:1. The molar concentration of pDNA phosphate is calculated by dividing the pDNA concentration (in mg/ml) by 330, the average nucleotide molecular mass. Liposomes (in SWFI) and pDNA (in 2x vehicle) were prepared at twice the final concentration in the formulation. An equal volume of liposomes was added to pDNA using a syringe and a 26 or 28 gauge needle. Liposomes were added in a steady stream, followed by brief, gentle vortex to mix (a few seconds on setting #4 of a Genie vortex mixer).

All cytofectin/co-lipid formulations used in this study remained uniformly opaque for several hours after preparation at room temperature without any visible aggregation. Formulations were injected 20 min – 1.5 hours after complexation. In a typical injection, where 5 μ g of pDNA was formulated with a cytofectin at 4:1 pDNA:cytofectin molar ratio, each muscle received 2.4 μ g cytofectin and 3.0 μ g neutral co-lipid in 50 μ l of vehicle. Even the highest pDNA+cytofecin:co-lipid dose tested in the mouse model (corresponding to 100 μ g VR4700 plasmid + 48 μ g GAP-

DMORIE + 60 µg DPyPE per mouse) did not appear to produce discomfort or result in any adverse reactions when injected into mouse muscle.

Preparation of pDNAs

5 The VR4700 plasmid was prepared using standard techniques known in the art. Briefly, VR1255, an optimized plasmid encoding firefly luciferase (Hartikka, J., *et al.*, *Human Gene Therapy* 7, 1205-1217 (1996)), had the coding sequence for influenza nuclear protein (NP) inserted in place of the luciferase coding sequence. The influenza nuclear protein sequence was derived from a plasmid termed nCMVint-tpaPRNP (Vahlsing, L., *et al.*, *J. Immunol. Methods* 174, 11-22 (1994)). More specifically, the VR4700 plasmid was created via the following procedure. The VR1255 plasmid was digested with Acc I + Bam HI, then the ends were blunted with Klenow, thus affording the desired vector fragment. The nuclear protein coding sequence was obtained by digesting nCMVintTPAPRNP with Acc I + Eco RI, and blunting the ends with Klenow. Both the vector fragment and the insert fragment were purified, then ligated with T4 DNA ligase. The ligation products were transformed in *E. coli* to kanamycin resistance, after which suitable plasmid bearing clones were identified based on restriction digest profiles. Standard cell culture techniques were used to expand a suitable clone, from which the plasmid was initially isolated and purified using well known, commercially available technology (Qiagen, Valencia, CA).

VR1412 LacZ plasmid was constructed by subcloning a cytoplasmic-targeted β-galactosidase gene into the VR1012 vector (Doh, S.G., *et al.*, *Gene Therapy* 4(7), 268-263 (1997)). The VR1012 backbone vector contains the human cytomegalovirus (CMV) immediate early 1 promoter/enhancer, CMV intron A, bovine growth hormone terminator and kanamycin resistance gene (Hartikka, J., *et al.*, *Human Gene Therapy* 7(10), 1205-17 (1996)).

VR5900 is a pDNA encoding hen egg lysozyme. For construction of this pDNA, gallus lysozyme cDNA was synthesized with overlapping oligonucleotides using Deep Vent DNA polymerase (NEB, Boston, MA). The nucleotide sequence

was obtained from GENBank, accession V00428. The sequence was humanized with the OLIGO 5.0 program and the corresponding oligonucleotides purchased from Retrogen (San Diego, CA). The PCR product was cloned into pCRII Blunt Topo (Invitrogen, Carlsbad, CA), sequenced in its entirety and subcloned into VR1055.

VR1055 is a Vical CMV promoter/enhancer-based expression vector that is identical to VR1012 except for the use of a minimal rabbit β -globin terminator in VR1055 (Hartikka, J., *et al.*, *Human Gene Therapy* 7, 1205-17 (1996)). HEL expression was confirmed by western blot with a rabbit anti-egg white lysozyme (Biodesign, Kennebunk, ME).

VR1904 is a pDNA encoding human factor IX. For construction, the factor IX cDNA insert from plasmid GT50 (kindly provided by Steven Josephs of Baxter Healthcare Corp., Round Lake, IL) was subcloned into the VR1012 vector.

VR1623 expresses a chimeric immunoglobulin with mouse variable regions fused to human constant regions. Human kappa and gamma (IgG1) constant regions were PCR amplified from human peripheral blood lymphocytes and cloned into VR1031, a bicistronic vector derived from VR1012 by insertion of a CITE sequence. This new construct was designated as VR1605. The variable region sequences from 38c13, a murine B-cell lymphoma (Bergman and Haimovich, 1977), were amplified by PCR from the plasmid pId (Tao and Levy, 1993, kindly provided by Dr. Ronald Levy, Stanford University Medical Center, CA) and cloned into VR1605 to make VR1623.

Bulk pDNA preparation and purification

Plasmid DNA was transformed into *Escherichia coli* DH10B or *Escherichia coli* DH5 α competent cells and grown in Terrific Broth (Sambrook, J., *et al.*, in *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, p. A.2 (1989)) supplemented with 50 mg/ml kanamycin in a 1 L shaker flask. Cells were harvested by centrifugation at the end of the exponential growth phase (approximately 16 hr), typically yielding 10 grams of biomass net weight per liter. Covalently closed circular pDNA was isolated by a modified lysis

procedure (Horn, N.A., *et al.*, *Human Gene Therapy* 6, 565-573 (1995)) followed by standard double CsCl-ethidium bromide gradient ultracentrifugation with an average yield of approximately 5 mg per liter. Plasmids were ethanol precipitated and resolubilized in saline at 4°C and dialyzed against saline. Endotoxin content was determined by the *Limulus* Amebocyte Lysate assay (Associates of Cape Cod, Inc., Falmouth, MA). All plasmid preparations were free of detectable RNA. Endotoxin levels were less than 7.0 Endotoxin Units/mg of plasmid DNA. The spectrophotometric A_{260}/A_{280} ratios were between 1.75 and 2.0. Plasmids were ethanol precipitated and resuspended in the injection vehicle at 4°C until completely dissolved. DNA was stored at -20°C until use.

Animal immunizations

The quadriceps muscles of restrained awake mice (female 8 - 12 week old BALB/c mice from Harlan Sprague Dawley, Indianapolis, IN) were injected with pDNA in 50 µl of vehicle using a disposable insulin syringe and a 28 gauge 1/2 inch needle (Becton-Dickinson, Franklin Lakes, NJ, Cat. No. 329430) fitted with a plastic collar cut from a micropipette tip. The collar length was adjusted to limit the needle tip penetration to a distance of about 2 mm into the central part of the rectus femoris muscle. Injection fluids and syringes were equilibrated to room temperature and the injection of a single 50 µl volume was carried out in 1-2 seconds.

Ketamine/xylazine anesthetized female New Zealand White rabbits (5 - 6 months of age, approximately 3 kg) were injected in the quadriceps muscle with 150 µg pDNA in 300 µl PBS using a 22 gauge 1 inch needle. Before injections, the injection site was shaved and cleaned with alcohol. The needle-free injection device, Biojector®2000 (Bioject Inc., Portland, OR), was tested in rabbits. The Biojector®2000 is a CO₂ powered jet injection system. In a pilot experiment, it was confirmed that the Biojector®2000 can deliver Indian ink solution through skin and into muscle tissue.

Animal care throughout the study was in compliance with the "Guide for the Use and Care of Laboratory Animals", Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, National Academy Press, Washington, D.C., 1996 as well as with Vical's Institutional Animal Care and Use Committee.

Anti-NP ELISA

Ninety-six well plates (Corning Incorporated, Cat. No. 3690, Corning, NY) were coated with 71-125 ng/well of influenza A/PR/8/34 nucleoprotein (NP) purified from recombinant baculoviral extracts in 100 µl BBS (89 mM Boric Acid + 90 mM NaCl + 234 mM NaOH, pH 8.3). The plates were stored overnight at +4°C and the wells washed twice with BBST (BBS supplemented with 0.05 % Tween 20, vol/vol). The wells were then incubated for 90 minutes with BB (BBS supplemented with 5 % nonfat milk, wt/vol) and washed twice with BBST again. Two-fold serial dilutions of mouse or rabbit serum in BB, starting at 1:20, were made in successive wells and the solutions were incubated for 2 hours at room temperature. Wells were then rinsed four times with BBST. Sera from mice hyperimmunized with VR4700 NP plasmid DNA were used as a positive control and pre-immune sera from mice and rabbits were used as negative controls.

To detect NP-specific antibodies, either alkaline phosphatase conjugated goat anti-mouse IgG-Fc (Jackson ImmunoResearch Laboratories, Cat. No. 115-055-008, West Grove, PA) or goat anti-rabbit IgG-Fc (Jackson ImmunoResearch Laboratories, Cat. No. 111-055-008, West Grove, PA) diluted 1: 5000 in BBS was added at 50 µl/well and the plates were incubated at room temperature for 2 hours. After 4 washings in BBST, 50 µl of substrate (1 mg/ml p-nitrophenyl phosphate, Calbiochem Cat. No. 4876 in 50 mM sodium bicarbonate buffer, pH 9.8 and 1 mM MgCl₂) was incubated for 90 min at room temperature and absorbance readings were performed at 405 nm. The titer of the sera was determined by using the reciprocal of the last dilution still giving a signal two times above background. Background was established using pre-immune serum diluted 1:20.

Splenocyte ^{51}Cr release assays

Single cell suspensions of splenocytes were pelleted and resuspended in RPMI 1640 medium containing L-glutamine and 25 mM HEPES and supplemented with penicillin (100 U/ml), streptomycin (100 $\mu\text{g}/\text{ml}$), 55 μM β -mercaptoethanol and 10 % FBS. Unless otherwise noted, all tissue culture media and reagents were obtained from Gibco BRL Life Technologies (Rockville, MD). Then, 2.5×10^7 splenocytes were cultured for 5 days in 25 cm^2 tissue culture flasks in a total of 10 ml of media with NP₁₄₇₋₁₅₅ peptide (H-2K^d TYQRTRALV) or β -gal₈₇₆₋₈₈₄ peptide (H-2L^d TPHPARIGL) at 1 $\mu\text{g}/\text{ml}$ and recombinant murine IL-2 (Roche Molecular Biochemicals, Indianapolis, IN) at 0.5 U/ml.

For the CTL assay, P815 cells were labeled with 0.15 mCi $\text{Na}_2^{51}\text{CrO}_4$ (NEN Life Science Products, Boston, MA) in 30 μl saline at 37°C for 35 minutes. Labeled cells were pulsed with 20 μg NP peptide or β -gal peptide (H-2L^d TPHPARIGL) in 1 ml RPMI 1640 media at 37°C for 40 minutes or were used unpulsed. Duplicate titrations of splenocytes were prepared by serially diluting the cells 1:3 in 96 well round bottom plates (ICN Biomedicals, Aurora, OH). Target cells were added at 1×10^4 cells/well in a final volume of 200 $\mu\text{l}/\text{well}$ at the designated effector:target ratios (E:T). The plates were centrifuged and incubated for 4 hours at 37°C with 5 % CO_2 . Counts per minute were determined for 100 μl of supernatant from each well. Specific lysis was calculated as % specific lysis = $[(a-b)/(c-b)]100$ where a is the average cpm released in the presence of effectors, b is the average cpm released from target cells incubated in media only and c is the cpm released from target cells in the presence of 1% Triton-X 100.

Example 1

GAP-DMORIE/co-lipid Enhances the Humoral Immune Response to pDNA-encoded Influenza Nucleoprotein (NP) in Mice

5 The present example demonstrates a quantitative comparison of the effects of the administration of various GAP-DMORIE:co-lipid complexes with pDNA versus pDNA alone in providing anti-NP antibody responses.

Transfection of muscles with pDNA encoding an immunogen elicits both humoral and cellular immune responses. To determine the extent of transfection
10 augmentation in an assay evaluating humoral immune response, changes in anti-NP antibody levels subsequent to immunization with an immunogen-encoding pDNA alone, and the same pDNA complexed with various adjuvant compositions, were quantified. The general features of the immunization assay are essentially as described by Ulmer *et al.* (*Science*, 259, 1745-1749 (1993)) and uses standard ELISA
15 technology to quantify antibody titers.

Mice were immunized using pDNA encoding influenza nuclear protein (NP), complexed with cytofectins formulated as a 1:1 (mol:mol) mixture with a co-lipid. The cytofectins were analyzed at a pDNA/cytofectin molar ratio of 4:1. Each animal in the test group (five animals per group) was injected with 5 µg pDNA in 50 µl
20 physiological saline (0.9% NaCl weight/volume in water) per leg in the rectus femoris muscle (10 µg pDNA total per animal) alone or as a cytofectin:co-lipid complex. Injections were performed at day "0" and at 3 weeks.

Cytofectin:co-lipid immune response enhancement was analyzed based on the ratio of the geometric mean titer (GMT) from a cytofectin-augmented transfection
25 group divided by the GMT from pDNA administration alone (see FIGS. 3 and 4). As shown in FIGS. 3 and 4, the preferred cytofectin GAP-DMORIE, when coupled with a co-lipid (especially DOPE or DPyPE), markedly enhances antibody responses to the encoded immunogen over both pDNA alone and pDNA complexed with other cytofectin:co-lipid combinations. Most surprisingly, the murine anti-NP antibody
30 titers at six weeks post-i.m. injection of VR4700 (FIG. 4) complexed with GAP-DMORIE:DPyPE resulted in a 10-fold increase in geometric mean anti-NP titer.

Example 2

GAP-DMORIE/DPyPE Enhances the Humoral Immune Response to pDNA-Encoded Influenza Nucleoprotein (NP) in Mice

5 The purpose of the present example is to demonstrate the ability of the preferred cytofectin:co-lipid, GAP-DMORIE/DPyPE, to enhance the humoral immune response to pDNA-encoded NP antigen. The most preferred cytofectin:co-lipid mixture is GAP-DMORIE/DPyPE at a 1:1 molar ratio. Rather than employing
10 the more cumbersome formal chemical nomenclature and stipulating the specific molar ratio for the mixture, this novel formulation has been named "Vaxfectin."

β -galactosidase assay

15 The muscle tissues were harvested, pulverized and extracted as previously described (Manthorpe, M., *et al*, *Gene Quantification*. Boston, Birkhauser 343-368 (1998)). The level of β -galactosidase expression in muscle extracts was quantified using a chemiluminescent assay according to the manufacturer's instructions (Boehringer Mannheim, Cat. No. 1758241, Indianapolis, IN). A standard curve,
20 prepared in pooled extract from uninjected muscles, was included on each plate using the β -galactosidase enzyme standard included in the kit.

Quantitation of anti-NP specific antibody secreting cells by ELISPOT assay

25 Anti-NP specific antibody secreting cells were quantified by the ELISPOT method using a previously described protocol (Slifka, M.K., *et al*, *J.Virol.* 69(3), 1895-1902 (1995)). Cells obtained from bone marrow (femur and tibia) were treated with 0.83 % NH_4Cl to lyse red blood cells. Cells were then resuspended in RPMI 1640 medium containing 5 % fetal calf serum (Hyclone, Logan, Utah), L-glutamine, HEPES, penicillin and streptomycin (LTI, Bethesda, Maryland). Nitrocellulose-
30 bottom 96-well Multiscreen filtration plates (Millipore Corporation, San Francisco, CA) were coated with 100 μl per well of 5 $\mu\text{g/ml}$ of NP antigen (influenza

nucleoprotein strain A/PR/8/34) in PBS and incubated overnight at 4°C. Plates were blocked with RPMI 1640 containing 5 % FBS for 2 h at room temperature. Blocking medium was replaced with 100 µl/well of blocking medium containing bone marrow cell suspension obtained from mice immunized with pDNA encoding influenza NP (with or without Vaxfectin), starting at 10⁶ cells, then diluted threefold row-wise down the plate. Control wells contained cells obtained from naïve mice diluted as above (earlier controls included an irrelevant antigen). Plates were incubated for 5 h at 37°C in a 7 % CO₂ humidified incubator. The plates were washed six times and incubated overnight at 4°C with 100 µl per well of biotinylated horse anti-mouse IgG (H+L, 1/1000 dilution, Vector Laboratories, Burlingame, CA) in PBS-T containing 1% FBS. Plates were further incubated for 1 h at room temperature with 100 µl/well of 5 µg/ml of horseradish peroxidase-conjugated avidin D (Vector Laboratories, Burlingame, CA). Antibody secreting cells were detected by adding 100 µl per well of substrate (3-amino-9-ethylcarbazole and H₂O₂) to the plates for 3 – 5 minutes. The reaction was terminated by washing profusely with tap water. Spots were counted under a dissecting microscope. Anti-NP specific antibody secreting cells were represented as number of spots per 10⁶ bone marrow cells.

Statistical evaluations

All statistical comparisons were performed using the non-parametric Mann-Whitney rank sum test (SigmaStat version 2.03, Jandel Scientific Software, San Rafael, CA). Differences were considered statistically significant when the p value was less than 0.05.

pDNA / Vaxfectin dose response

To compare the effects of increasing pDNA dose, and the effect of boost injections, mice were given bilateral i.m. injections of 1 µg, 5 µg or 25 µg of naked VR4700 plasmid per muscle (thus affording a total pDNA dose of 2, 10 and 50 µg per animal, respectively) at three-week intervals. The results are shown in Fig. 5. Higher anti-NP titers were reached when more naked pDNA was injected per muscle, and

titers increased after the first and the second boost injections. However, no further increase in anti-NP titers was observed with any of the pDNA doses when a third boost injection was given at 9 weeks (data not shown), suggesting that plateau antibody titer levels had been reached with naked pDNA.

5 A second set of mice received equivalent pDNA doses formulated with Vaxfectin. The results are shown in Fig. 5. Here, a 7- to 20-fold increase in antibody titers with all three pDNA doses was observed. The highest average anti-NP titers per group in this experiment ($204,800 \pm 56,087$, $n = 5$ mice) were measured at 9 weeks with 25 μ g pDNA dose formulated with Vaxfectin. As was seen with naked pDNA
10 injections, no further increase in anti-NP titers was observed with any of the Vaxfectin groups when a third boost injection was given at 9 weeks (data not shown). Thus, Vaxfectin enhanced antibody titers to levels that could not be reached with naked pDNA alone, either by increasing the pDNA dose or the number of injections. The most striking finding was that as little as 1 μ g of pDNA per muscle formulated with
15 Vaxfectin resulted in up to 5-fold higher anti-NP titer than that obtained with 25 μ g naked VR4700 alone.

A separate experiment was done to address whether multiple bilateral injections are required to obtain Vaxfectin-mediated enhancement in humoral immune response. The results are shown in Table 1. Formulating 5 μ g VR4700 pDNA with
20 Vaxfectin produced a significant 6-fold increase in anti-NP titers 20 days after a single unilateral i.m. injection in mice, indicating that Vaxfectin can enhance antibody response after a single dose.

TABLE 1

Antibody titers in mouse serum after a unilateral i.m. injection of pDNA coding for influenza nuclear protein (NP) protein^a.

	Average anti-NP titers		
	pDNA	Vaxfectin	Fold Increase
Day 20	710 ± 162	4,573 ± 1,243 ^b	6 x
Day 42	5,387 ± 767	35,200 ± 6,096 ^b	7 x

^aMice received a single injection of 5 µg naked VR4700 plasmid in 50 µl of 150 mM NaP in the right quadriceps muscle. A second group of mice received 5 µg VR4700 formulated with Vaxfectin at a pDNA:cationic lipid molar ratio of 4:1. On day 21, mice were given a single boost injection in the same muscle. Total NP-specific IgG antibody titers were determined from serum samples on day 20 and 42 (average ± S.E.M., n = 15 mice).

^bSignificantly different from naked pDNA control value (p < 0.01, Mann-Whitney rank sum test).

Formulation optimization

Different pDNA:cationic lipid ratios were tested in the murine immunization model to optimize Vaxfectin formulations. The results, shown in Fig. 6, indicate that injecting more pDNA-Vaxfectin complex (thus, increasing both the amount of plasmid and the amount of Vaxfectin simultaneously) increased antibody titers in a dose dependent manner. This trend was observed for both the 2:1 and 4:1 pDNA:cationic lipid molar ratios. When the same 5 µg pDNA dose was injected with increasing amount of Vaxfectin (thus decreasing the pDNA:cationic lipid molar ratio), antibody titers again increased in a Vaxfectin dose dependent manner (Fig. 6B). Higher pDNA doses were also examined, but injecting 50 µg pDNA per limb formulated with Vaxfectin at 4:1 pDNA:cationic lipid molar ratio did not produce any

further increase in anti-NP titers, compared to 25 µg pDNA formulated with Vaxfectin at the same 4:1 ratio (data not shown).

Duration of enhanced humoral response

To investigate the duration of the Vaxfectin-enhanced humoral response, NP specific antibody titers were followed for nine months after initial injection in the murine vaccination model. The results are shown in Fig. 7A. Three weeks after the boost injection given on day 21, anti-NP titers in the Vaxfectin group were 9-fold higher than in the naked pDNA control group. During the subsequent weeks, antibody titers in the Vaxfectin group gradually declined but remained significantly higher than in the controls throughout the course of the experiment. Forty weeks after the start of the experiment, anti-NP titers in Vaxfectin group were still 4-fold higher than in the naked pDNA group.

In a parallel experiment, another set of mice received a boost injection on week 3, and a second identical boost at 3 months. The results are shown in Fig. 7B. The second boost injection increased antibody titers in both groups by 2- to 3-fold. However, anti-NP titers in the Vaxfectin group appeared to remain at these elevated levels for several months, whereas the naked pDNA group yielded titers comparable to those after a single boost at the end of the experiment. Consequently, nine months after the start of the experiment, anti-NP titers in Vaxfectin group were 17-fold higher than in the pDNA control group.

Vaxfectin maintains a strong CTL response

It would be highly desirable that an adjuvant used in combination with pDNA vaccines to enhance humoral immune response would not at the same time diminish cell-mediated immunity. To evaluate this, CTL assays were performed after mice had been injected with various doses of pDNA with or without Vaxfectin. The results are shown in Fig. 8. Vaxfectin did not have a significant effect on CTL response when formulated at different pDNA and cationic lipid ratios (Fig. 8A), after a single boost (Fig. 8C) or multiple boost injections (Fig. 8A and 8B), or when delivered in PBS

(Fig. 8A) or 150 mM NaP vehicle (Fig. 8C). Injecting 25 µg of naked pDNA per muscle appeared to result in stronger CTL responses than 1 µg pDNA dose. Again, Vaxfectin did not have a significant effect on CTL response with either pDNA dose (Fig. 8B). Taken together, these results show that Vaxfectin could be used to enhance humoral immune response with pDNA vaccines while maintaining the strong CTL response characteristic of pDNA immunization.

Vaxfectin does not increase muscle transfection

To elucidate the mechanism by which Vaxfectin enhances antibody responses, the effect of Vaxfectin on muscle expression *in vivo* was studied. In these experiments, pDNA (VR1412) encoding β-galactosidase was injected either alone or formulated with Vaxfectin and individual muscles were periodically assayed for reporter gene expression. The results are shown in Fig. 9. One day after injections, β-galactosidase expression in both groups was the same, indicating that Vaxfectin did not affect the initial transfection of muscle with pDNA. Between day 1 and 7, muscle expression in the naked pDNA group increased 7-fold. In contrast, expression in the Vaxfectin group decreased by 25% during the same time period. Between day 7 and 21, reporter gene levels remained the same in the naked pDNA group, whereas β-galactosidase expression in muscle continued to decline in the Vaxfectin group and was more than 20-fold lower than in the pDNA control group at day 21. Thus, at later time points, transgene expression in muscle was markedly reduced in the Vaxfectin group, whereas antibody levels were higher. This lack of correlation between muscle expression and antibody titers indicates that Vaxfectin mediated enhancement in antibody response cannot be explained by facilitated transfection of myofibers and/or increased synthesis of the antigen in muscle tissue.

The mechanism by which Vaxfectin enhances the antigen-specific antibody response is unclear. It is possible that Vaxfectin delivers the pDNA to multiple cell types within muscle tissue, including antigen-presenting cells, whereas needle injection of pDNA without Vaxfectin might principally transfect muscle fibers. Alternatively, the pDNA-lipid complex may be better able to exit the muscle and

transduce distal tissue, including cells in the regional draining lymph nodes. Vaxfectin could protect the plasmid against nucleases, enabling the pDNA-lipid complex to reach tissues distant from the injection site. Vaxfectin may also induce inflammation, resulting in the damage of many transduced muscle fibers and thereby releasing more soluble antigen soon after injection. A decrease in antigen production in the following days may select for higher affinity antigen specific B cells by limiting antigen, resulting in an increase in antibody titers.

Vaxfectin increases the number of antigen-specific plasma cells in bone marrow

Elevated anti-NP titers in Vaxfectin treated animals were maintained for several months after the boost injection (Fig. 7). Since long-lived plasma cells in bone marrow have been shown to be the major mechanism for maintaining persistent antibody production after viral infection (Slifka, M.K., *et al*, *J.Virol.* 69(3), 1895-1902 (1995), Slifka, M.K., *et al*, *Curr. Opin. Immunol* 10(3), 252-258 (1998)) the number of anti-NP antibody secreting cells from bone marrow was quantified using an ELISPOT assay. The results showed that Vaxfectin produced a statistically significant 3- to 5-fold increase in the number of NP specific plasma cells in bone marrow. Furthermore, antibody titers in individual mice roughly correlated with the number of anti-NP antibody secreting cells in bone marrow, both in the naked pDNA and in the Vaxfectin groups (Table 2).

TABLE 2

Quantitation of anti-NP antibody secreting cells in bone marrow by ELISPOT assay

	PDNA			Vaxfectin		
	Mouse	Anti-NP	SFC per	Mouse	Anti-NP	SFC per
		Titer	10 ⁶ cells		titer	10 ⁶ cells
Experiment 1^a	1	3,200	1.8	1	51,200	17.3
	2	12,800	5.0	2	102,400	11.8
	3	25,600	8.3	3	102,400	29.8
	4	51,200	13.7	4	204,800	21.2
				5	204,800	34.8
	Average	23,200	7.2	Average	133,120	23.0^b
Experiment 2^c	1	12,800	11.0	1	51,200	39.5
	2	12,800	15.0	2	102,400	35.0
	3	25,600	12.5	3	204,800	85.2
	4	51,200	21.3	4	204,800	132.8
	Average	25,600	14.9	Average	140,800	73.1^d

5 ^aMice received bilateral intramuscular injections of 5 µg VR4700 pDNA in 50 µl PBS, either alone or formulated with Vaxfectin at 4:1 pDNA:cationic lipid molar ratio. Identical boost injections were given at three weeks and at three months. Mice were sacrificed four months after the start of the experiment (one month after the second boost injection). Antibody titers were measured from terminal bleeds and the number of anti-NP specific spot forming cells (SFC) per 10⁶ bone marrow cells were quantified.

10 ^bSignificantly different from pDNA control value (p = 0.032, Mann-Whitney rank sum test).

^c Mice received bilateral intramuscular injections of 5 µg VR4700 pDNA in 50 µl PBS, either alone or formulated with Vaxfectin at 4:1 pDNA:cationic lipid molar ratio. Identical boost injections were given at three weeks and at nine months. Mice were sacrificed eleven months after the start of the experiment (two months after the second boost injection). Antibody titers were measured from terminal bleeds and anti-NP secreting cells were quantified from bone marrow.

^d Significantly different from pDNA control value ($p = 0.029$, Mann-Whitney rank sum test).

The data from the ELISPOT assays indicate that the use of Vaxfectin increases the number of antigen specific plasma cells in the bone marrow. This increase in plasma cells may be due to the adjuvant properties of the pDNA-lipid complexes. Injection of blank pDNA complexed with a cationic lipid into the peritoneum of murine ovarian tumor bearing C3H/HeN mice induces the production of IL-6, IFN- γ , and TNF- α (Horton, H.M., *et al*, *J. Immunol.* 163(12), 6378-6385 (1999)). These cytokines were not induced in mice treated with pDNA or lipid only, suggesting that the pDNA-lipid complexes are immunostimulatory *in vivo*. The immunostimulatory properties of pDNA-lipid complexes were also reported for experiments in which mice were injected intravenously with pDNA complexed with cationic lipid (Dow, S.W., *et al*, *J. Immunol.* 163(3), 1552-1561 (1999)). As for intraperitoneal and intravenous injection of pDNA-lipid complexes, intramuscular injection of pDNA-Vaxfectin may also induce cytokines, including IL-6, a cytokine that promotes the differentiation of activated B cells to plasma cells. Thus, the pDNA-Vaxfectin complexes may indirectly enhance antibody titers by increasing the number of antibody producing B cells.

It is also possible that components of Vaxfectin might mimic naturally occurring mitogens that can directly stimulate polyclonal expansion of B cells. This could enhance the specific immune response against the transgene expressed by the muscle cells by increasing the number of responding B cells. Thus, increased transfection of APCs or delivery of pDNA to the draining lymph nodes with transfection of cells in the lymph nodes, muscle damage resulting in increased availability of soluble antigen and the immunostimulatory properties of the pDNA-Vaxfectin complexes could each contribute to the adjuvant effect of Vaxfectin.

Example 3

Vaxfectin Enhances Antibody Titers in Rabbits

The purpose of the present example is to demonstrate the adjuvant effect of
5 GAP-DMORIE:co-lipids (e.g., Vaxfectin) in rabbits when formulated in
polynucleotide-based vaccines.

Female New Zealand white rabbits (5 - 6 months old) were anesthetized, then
injected in a hind leg with 300 μ l of a PBS solution containing either 150 μ g of
VR4700 plasmid DNA or a PBS solution containing a complex of 150 μ g VR4700
10 plasmid with GAP-DMORIE:DPyPE (1:1) prepared at a 4:1 mol:mol pDNA:GAP-
DMORIE ratio. Each rabbit received a single injection using a sterile disposable,
plastic insulin syringe and 22 gauge 1 inch needle at day zero, plus an identical
“boost” injection in the opposite hind leg at 6 weeks. The animals were bled through
an ear vein prior to immunization, and at weeks 3, 6, 7, 9, and 13 . The six-week
15 bleed was performed the same day, but before boost injection was given.

Using a single unilateral i.m. injection performed with needle and syringe,
Vaxfectin produced a robust 20-fold increase in antibody titers at three weeks
compared to injection of naked pDNA. The results are shown in Fig. 10. After a
boost injection given at 6 weeks, anti-NP titers in both groups increased
20 approximately by an order of magnitude, with antibody titers in the Vaxfectin group
remaining 20- to 50-fold higher than in the naked pDNA group throughout the course
of the experiment. When rabbits were immunized with the Biojector®2000 device,
Vaxfectin did not appear to enhance antibody response after a single unilateral
injection. After a boost injection was given at 6 weeks, anti-NP titers in the Biojector
25 Vaxfectin group were up to 8-fold higher than in the corresponding naked pDNA
group.

Example 4

Vaxfectin Enhances Antibody Production and Promotes TH1 Type Immune Response to Various Plasmid DNA-encoded Antigens

The purpose of the present example is to demonstrate the adjuvant effect of GAP-DMORIE:co-lipid (e.g., Vaxfectin) when formulated with various model antigens, and to further characterize the immune responses to pDNA formulations containing GAP-DMORIE:co-lipid.

Immunization and serum collection

Restrained, awake mice received 5 µg of pDNA encoding A/PR/8/34 NP (VR4700), hen egg lysozyme (HEL, VR5900), *E.coli* Lac Z (β-gal, VR1412), mouse Id/human Fc (immunoglobulin variable regions from 38C13, a murine lymphoma cell line fused to a human IgG1 constant region, VR1623), or human factor IX (VR1904) prepared in PBS with and without Vaxfectin (50 µl) and injected into the rectus femoris of 8-10 week old female mice. Mice were boosted at 3 weeks with the same dose and formulation. Mice were bled from the ophthalmic venous plexus prior to the first injection, 1 day prior to the boost, and at 6 weeks following the first injection.

IgG antibody ELISAs

Antibody titers were determined by coating 96 well, ½ area flat well microtiter plates (Corning/Costar, Inc., Corning, NY) with 0.035 µg influenza nucleoprotein (purified from recombinant baculoviral extracts), 0.25 µg hen egg lysozyme (HEL, Sigma, St. Louis, MO), 0.25 µg *E.Coli* β-galactosidase (β-gal, Sigma, St.Louis, MO), 2.2 µg mouse Id (Southern Biotech, Birmingham, AL), or 0.3 µg human Factor IX (Calbiochem, La Jolla, CA) in 50 µl BBS (89 mM Boric Acid, 90 mM NaCl pH 8.3) per well. Plates were incubated overnight at 4°C then washed 4 times with BBST (BBS with 0.1% Tween 20). NP coated wells were blocked with 100 µl of NP assay buffer (5% nonfat milk in BBS) and wells of all other plates were blocked with 100 µl

of BSA assay buffer (1% bovine serum albumin in BBS) for 1 hour at room temperature. Two-fold serial dilutions of sera in assay buffers, starting at 1:25, were prepared and 50 µl aliquots added to each well. Following a 2-hour incubation at room temperature and 4 washes, alkaline phosphatase conjugated goat anti-mouse IgG-Fc (Jackson ImmunoResearch, West Grove, PA) diluted 1:5000 in assay buffer was added at 50 µl/well. The plates were incubated for 2 hours at room temperature, washed 4 times and 50 µl of p-NPP substrate (1 mg/ml para-nitrophenyl phosphate, Calbiochem, La Jolla, CA, in 50 mM sodium bicarbonate buffer, pH 9.8 and 1 mM MgCl₂) was added per well. The absorbance at 405 nm was read after 1.5 hours at room temperature. The titer is the reciprocal of the last dilution with a signal 2 times that of pre-bleed samples.

Antigen specific IgG1 and IgG2a ELISAs

Alkaline phosphatase conjugated, mouse sub-isotype specific monoclonal antibodies were pre-titrated with standards to determine the dilution at which equal absorbance values were obtained for equal amounts of standard. For the titrations, plates were coated overnight at 4°C with 0.1 µg/50 µl/well of affinity purified goat anti-mouse kappa antisera in BBS. Plates were washed and blocked as for the NP ELISA described above. Purified mouse IgG1, κ or IgG2a, κ were serially diluted and added to the plates at 50 µl/well. After incubating for 2 hours at room temperature, alkaline phosphatase conjugated rat anti-mouse IgG1 and IgG2a (Pharmingen, La Jolla, CA) were serially diluted and added to washed plates. The assay was completed as for the NP antibody ELISA. The assay for measurement of antigen specific sub-isotype serum titers was as described for total IgG levels with the following modifications: the alkaline phosphatase conjugated anti-mouse IgG1 and anti-mouse IgG2a were diluted 1:1500 and 1:200 respectively.

Stimulation of CTL

Spleens were removed from euthanized mice at 11-12 weeks after the first injection, and 2.5×10^7 splenocytes were cultured for 5 days in 6 well plates in a total

of 5 ml of RPMI 1640 medium (unless otherwise noted, all tissue culture reagents were obtained from Gibco BRL Life Technologies, Rockville, MD) containing L-glutamine and 25 mM HEPES and supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), 5.5×10^{-5} M β -mercaptoethanol and 10% FBS (10%

- 5 media) with either NP₁₄₇₋₁₅₅ peptide (H-2K^d TYQRTRALV) or β -gal₈₇₆₋₈₈₄ peptide (H-2L^d TPHPARIGL) at 1 µg/ml and recombinant murine IL-2 (Roche Molecular Biochemicals, Indianapolis, IN) at 0.5 U/ml.

⁵¹Cr release assay

- 10 To detect antigen specific lysis, P815 cells were labeled with 0.15 mCi Na₂⁵¹CrO₄ (NEN Life Science Products, Boston, MA) and either pulsed with 20 µg NP₁₄₇₋₁₅₅ peptide or 50 µg β -gal₈₇₆₋₈₈₄ peptide in 1 ml RPMI 1640 media or were used unpulsed. Duplicate aliquots of stimulated splenocytes were serially diluted in 96 well round bottom plates (ICN Biomedicals, Aurora, OH) and target cells were added
- 15 at the designated effector:target ratios in a final volume of 200 µl/well. The plates were centrifuged and incubated for 4 hours at 37°C with 5% CO₂. After incubation, 100 µl of supernatant from each well was analyzed. Specific lysis was calculated as % specific lysis = [(a-b)/(c-b)]100 where a is the average cpm released in the presence of effectors, b is the average cpm released from target cells incubated in media only
- 20 and c is the cpm released from target cells in the presence of 1% Triton-X 100.

Cytokine profiles

- To determine cytokine secretion profiles of spleen cells re-stimulated *in vitro* with antigen, splenocytes were plated in duplicate at 4×10^5 cells/100µl/well in 96
- 25 well flat bottom culture plates with purified NP (purified from recombinant baculoviral extracts) or β -gal protein (Sigma, St.Louis, MO) at 5 µg/ml. Culture supernatants were harvested after 72 hours at 37°C with 5% CO₂. Cytokines in culture supernatants were quantified with a mouse IFN- γ ELISA kit (Pharmingen, La

Jolla, CA) and mouse IL-4 ELISA mini-kit (Endogen, Woburn, MA) according to the manufacturers' instructions.

Statistical analysis

5 Statistical analyses were performed with the 2-tailed student t-test.

Effect of Vaxfectin on antigen specific IgG titers

Antigen specific antibody titers for sera collected 1 day prior to the boost at 3 weeks, and for sera collected at 6 weeks following the first injection are shown in
10 Figure 11. Immunization with pDNA/Vaxfectin had a modest effect on the three-week titers of the anti-NP and anti-Factor IX antibodies and an even greater effect on the anti-mouse id and anti-HEL antibody titers. Vaxfectin had no effect on the serum titers of anti- β -gal antibodies at 3 weeks. Three weeks following the boost immunizations, titers for mice receiving pDNA/Vaxfectin were increased over those
15 receiving naked pDNA for all five of the antigens. Table 3 summarizes the antigen specific IgG responses at 6 weeks for all 5 model antigens. Vaxfectin increased the titers of pDNA induced anti-NP and anti-HEL antibodies 8 fold and 10 fold respectively over naked pDNA and increased the titers of anti- β -gal, anti- factor IX and anti-mouse Id 3 fold over naked pDNA.

Table 3. IgG Antibody titers at 6 Weeks*

Antigen	Ab Titer for Naked (average \pm std error)	Ab Titer for Vaxfectin (average \pm std error)	Fold Increase with Vaxfectin
Influenza NP	9,821 \pm 1,418	74,933 \pm 8,597	8 x
HEL	14,300 \pm 3,798	136,720 \pm 27,096	10 x
β -gal	27,280 \pm 4,017	69,760 \pm 12,544	3 x
Mouse Id/Human Fc	972 \pm 381	2,503 \pm 517	3 x
Human Factor IX	10,240 \pm 2,504	30,320 \pm 6,752	3 x

*Mice received a bilateral injection of 5 μ g pDNA +/- Vaxfection into each rectus femoris muscle at 0 and 3 weeks. Antibody titers were determined at 6 weeks (n = 20 for all groups, except for NP where n = 29 for Naked NP pDNA, n = 30 for NP pDNA/Vaxfectin, and for Mouse Idiotypic where n = 19 for Naked Mouse Id pDNA).

Effect of Vaxfectin on the CTL response

Plasmid DNA vaccination by the intramuscular route typically results in strong CTL responses to the encoded antigen (Ulmer *et al.*, 1993; Raz *et al.*, 1996; Donnelly *et al.*, 1997). One possible outcome of formulating pDNA with an adjuvant to boost antibody responses is induction of a Th2 type response which could result in a weaker cell mediated immune response. To determine the effect of Vaxfectin on the pDNA induced CTL response, spleens from mice immunized with NP or β -gal pDNA were harvested 8-9 weeks following the boost injection. Splenocytes cultured with

NP or β -gal peptide for 5-6 days were assayed for CTL lysis of P815 target cells pulsed with NP or β -gal peptide. Unpulsed P815 cells were used to detect non-specific lysis. The antigen specific CTL effector titration curves for % lysis of peptide pulsed target cells are shown in Figure 12. The results for both NP and β -gal pDNA indicate that formulation of pDNA with Vaxfectin has no significant effect on the CTL response at any of the effector:target ratios tested ($p > 0.05$ at all E:T ratios).

Effect of Vaxfectin on IgG1 and IgG2a antibody titers

The Th1 type immune responses induced by intramuscular pDNA immunization promote antibody heavy chain switch in responding B cells to the IgG2a sub-isotype (Raz et al., 1996). Thus production of antigen specific IgG2a is greater than antigen specific IgG1. The use of an adjuvant in pDNA vaccines could qualitatively change the immune response, resulting in greater production of either IgG1 or IgG2a. To determine the effect of Vaxfectin on the relative proportion of antigen specific serum IgG1 to IgG2a when formulated with various antigen plasmid DNAs, 6 week sera were analyzed for antigen specific sub-isotype titers.

As shown in Figure 13a, immunizations with naked pDNA encoding different antigens result in sub-isotype profiles that are unique to each antigen. Although the relative proportion of IgG1 and IgG2a varied for different antigens, IgG2a was the predominant sub-isotype produced, consistent with a Th1 type immune response. Vaxfectin formulated with all five model antigen pDNAs results in an increase of both antigen specific antibody sub-isotypes (Table 4). Increases in antigen specific IgG1 and IgG2a were approximately the same magnitude for Vaxfectin formulated pDNA for 4 of the model antigens. As compared to titers obtained with naked pDNA, formulating pDNA with Vaxfectin increased anti-HEL IgG1 titers 9-fold and IgG2a titers 11-fold. Vaxfectin increased anti- β -gal, anti-mouse Id/human Fc and anti-factor IX IgG1 titers 2 to 5-fold and IgG2a titers 2 to 4-fold over naked DNA. Vaxfectin formulated with NP pDNA increased the average anti-NP IgG1 antibody titer by 15-fold over naked pDNA. However, the average anti-NP IgG2a antibody titer was only increased 3-fold. Thus, the relative proportions of IgG1 and IgG2a elicited by

immunization of pDNA/Vaxfectin remains similar to the proportions generated when naked pDNA is used to immunize mice except in the case of NP pDNA/Vaxfectin (Figure 13b). In this case, there is a much greater increase in antibody titer of anti-NP IgG1 than anti-NP IgG2a. For all of the pDNAs formulated with Vaxfectin, titers of antigen specific IgG2a were higher than antigen specific IgG1, suggesting a Th1 type response.

Table 4. IgG1 and IgG2a antibody titers at 6 weeks*

Influenza NP		HEL		β -gal		Mouse Id/Human Fc		Human Factor IX	
Isotype	IgG1	IgG2a	IgG1	IgG2a	IgG1	IgG2a	IgG1	IgG2a	IgG1
Naked PDNA	11,172 $\pm 1,825$	213,628 $\pm 60,785$	14,210 $\pm 3,364$	25,620 $\pm 6,719$	8,620 $\pm 1,887$	356,480 $\pm 67,452$	493 ± 339	426 ± 228	6,700 $\pm 1,789$
PDNA/ Vaxfectin	171,467 $\pm 44,030$	745,813 $\pm 161,747$	128,340 $\pm 28,009$	280,720 $\pm 61,683$	16,100 $\pm 6,807$	944,640 $\pm 337,513$	878 ± 281	1,615 ± 638	35,380 $\pm 9,449$
Increase over Naked	15 X	3 X	9 X	11 X	2 X	3 X	2 X	4 X	5 X
									2 X

*Mice received a bilateral injection of 5 μ g pDNA +/- Vaxfectin into each rectus femoris muscle at 0 and 3 weeks. Antibody titers were determined at 6 weeks (n = 20 for all groups, except for NP where n = 29 for Naked NP pDNA, n = 30 for NP pDNA/Vaxfectin, and for Mouse Idiotype where n = 19 for Naked Mouse Id pDNA).

Effect of Vaxfectin on the cytokine profile

The antigen specific antibody sub-isotype analyses suggest that the responses induced with Vaxfectin formulated pDNA, as for naked pDNA are Th1 type responses. To confirm that Vaxfectin has no effect on the Th cytokine profile in an antigen specific *in vitro* recall response, spleens from groups of mice immunized with NP or β -gal pDNA formulated with or without Vaxfectin were harvested 8-9 weeks following the boost injection. Splenocytes were cultured and stimulated with NP or β -gal protein. Supernatants harvested from the cultured cells were assayed for IFN- γ and IL-4 production. Immunizations with NP or β -gal plasmid DNA formulated with or without Vaxfectin resulted in IFN- γ production in splenocyte cultures from all groups of immunized mice (Figure 14). Low levels of IL-4 were produced in all groups of mice; however, IFN- γ was the predominant cytokine produced, suggesting a Th1 biased response.

In summary, the foregoing examples demonstrate the robust adjuvant effects of a unique cationic lipid-based formulation for nucleic acid vaccines. The stimulation of the humoral response can be accomplished without diminishing the strong cytolytic responses typical of nucleic acid-based vaccines. The adjuvant activity is seen in both mice and rabbits, thus implying the pharmaceutical applications in other mammals, as well as offering potential benefit in nucleic acid-based preparation of monoclonal and polyclonal antibodies. GAP-DMORIE/co-lipid (e.g., Vaxfectin) mediated enhancement of the antibody responses was readily observed after a single unilateral intramuscular injection. This is important for the immunization of farm animals where single-shot vaccines are highly desirable since roundup of range animals is expensive and can result in loss of production due to stress (Beard, C.W., *et al*, *Nat. Biotechnol* 16(13), 1325-1328 (1998)).

Example 5

Human Administration

Immunogenic compositions comprising pDNA encoding hemagglutinin(HA), mixed with an adjuvant containing GAP-DMORIE formulated as a 1:1 (mol:mol)

mixture with DPyPE, are prepared according to the method described above. The pDNA/adjuvant molar ratio is 4:1. Three injections of 0.1, 0.5, 1.0, or 2.5 mg pDNA in physiological saline, as a complex with the adjuvant, are injected into humans at 4-week intervals in alternate deltoids. Serum is removed from the humans and the HA antibody levels are determined by serial dilution using a standard ELISA assay, as described above. Immune responses of the human subjects to the HA antibody are induced, as indicated by GMT antibody titer values.

Other Embodiments

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

What Is Claimed Is:

1. An adjuvant composition comprising GAP-DMORIE and one or more co-lipids.
- 5 2. The adjuvant composition of claim 1 wherein the co-lipid is a neutral lipid.
3. The adjuvant composition of claim 1 wherein the co-lipid is a phosphatidylethanolamine.
4. The adjuvant composition of claim 3 wherein the phosphatidylethanolamine is selected from the group consisting of DOPE, DPyPE, and DMPE.
- 10 5. The adjuvant composition of claim 4 wherein the phosphatidylethanolamine is DOPE.
6. The adjuvant of claim 4 wherein the phosphatidylethanolamine is DPyPE.
7. The adjuvant of claim 1 wherein the GAP-DMORIE and the co-lipid are in molar ratio of from about 9:1 to about 1:9.
- 15 8. The adjuvant of claim 1 wherein the GAP-DMORIE and the co-lipid are in molar ratio of from about 4:1 to about 1:4.
9. The adjuvant of claim 1 wherein the GAP-DMORIE and the co-lipid are in molar ratio of from about 2:1 to about 1:2.
10. The adjuvant of claim 1 wherein the GAP-DMORIE and the co-lipid are in molar ratio of about 1:1.
- 20 11. The adjuvant of claim 6 wherein the GAP-DMORIE and DPyPE are in molar ratio of from about 2:1 to about 1:2.
12. The adjuvant of claim 6 wherein the GAP-DMORIE and DPyPE are in molar ratio of about 1:1.
- 25 13. An immunogenic composition comprising an immunogen and an adjuvant composition comprising GAP-DMORIE and one or more co-lipids.
14. The immunogenic composition of claim 13 wherein the immunogen comprises an immunogen-encoding polynucleotide.
15. The immunogenic composition of claim 14 wherein the immunogen-encoding polynucleotide is DNA, RNA, or nucleic acid oligomer.
- 30

16. The immunogenic composition of claim 14 wherein the immunogen-encoding polynucleotide is a linear or circular polynucleotide.
17. The immunogenic composition of claim 14 wherein the immunogen-encoding polynucleotide is all or part of a plasmid DNA.
- 5 18. The immunogenic composition of claim 14 wherein the co-lipid is selected from the group consisting of DOPE, DPyPE, and DMPE.
19. The immunogenic composition of claim 18 wherein the co-lipid is DOPE.
20. The immunogenic composition of claim 18 wherein the co-lipid is DPyPE.
21. The immunogenic composition of claim 14 wherein the GAP-DMORIE and
10 the co-lipid are in molar ratio of from about 9:1 to about 1:9.
22. The immunogenic composition of claim 14 wherein the GAP-DMORIE and the co-lipid are in molar ratio of from about 4:1 to about 1:4.
23. The immunogenic composition of claim 14 wherein the GAP-DMORIE and the co-lipid are in molar ratio of from about 2:1 to about 1:2.
- 15 24. The immunogenic composition of claim 14 wherein the GAP-DMORIE and the co-lipid are in molar ratio of about 1:1.
25. The immunogenic composition of claim 20 wherein the GAP-DMORIE and DPyPE are in molar ratio of from about 2:1 to about 1:2.
26. The immunogenic composition of claim 20 wherein the GAP-DMORIE and
20 DPyPE are in molar ratio of about 1:1.
27. A method for immunizing a vertebrate comprising administering into a tissue or cavity of said vertebrate an immunogenic composition comprising one or more immunogen-encoding polynucleotide and an adjuvant composition comprising GAP-DMORIE, wherein an immunogen is expressed in the vertebrate in an amount
25 sufficient to generate an immune response to the immunogen.
28. The method of claim 27 wherein the immunogenic composition further comprises one or more co-lipids.
29. The method of claim 28 wherein the immunogen-encoding polynucleotide is DNA, RNA, or nucleic acid oligomer.

30. The method of claim 28 wherein the immunogen-encoding polynucleotide is all or part of a plasmid DNA.

31. The method of claim 28 wherein the co-lipid is selected from the group consisting of DOPE, DPyPE, and DMPE.

5 32. The method of claim 28 wherein the co-lipid is DOPE.

33. The method of claim 28 wherein the co-lipid is DPyPE.

34. The method of claim 28 wherein the GAP-DMORIE and the co-lipid are in molar ratio of from about 9:1 to about 1:9.

10 35. The method of claim 28 wherein the GAP-DMORIE and the co-lipid are in molar ratio of from about 4:1 to about 1:4.

36. The method of claim 28 wherein the GAP-DMORIE and the co-lipid are in molar ratio of from about 2:1 to about 1:2.

37. The method of claim 28 wherein the GAP-DMORIE and the co-lipid are in molar ratio of about 1:1.

15 38. The method of claim 33 wherein the GAP-DMORIE and DPyPE are in molar ratio of from about 2:1 to about 1:2.

39. The method of claim 33 wherein the GAP-DMORIE and DPyPE are in molar ratio of about 1:1.

40. The method of claim 27 wherein the vertebrate is a mammal.

20 41. The method of claim 40 wherein the mammal is a human.

42. The method of claim 28 wherein the immunogenic composition is a pharmaceutical composition.

25 43. The method of claim 28 wherein said immunogen is selected from the group consisting of a bacterial polypeptide, a fungal polypeptide, a parasite polypeptide, an allergenic polypeptide, a tumor specific polypeptide, an immunogenic fragments, derivatives, or analogs thereof.

44. The method of claim 28 wherein said tissue is selected from the group consisting of muscle, skin, brain tissue, lung tissue, liver tissue, spleen tissue, bone marrow tissue, thymus tissue, heart tissue, lymph tissue, blood tissue, bone tissue, 30 connective tissue, mucosal tissue, pancreas tissue, kidney tissue, gall bladder tissue,

stomach tissue, intestinal tissue, testicular tissue, ovarian tissue, uterine tissue, vaginal tissue, rectal tissue, nervous system tissue, eye tissue, glandular tissue, and tongue.

45. The method of claim 28 wherein said cavity is selected from the group consisting of lung, mouth, nasal cavity, stomach, peritoneum, intestine, heart
5 chamber, vein, artery, capillary, lymphatic, uterus, vagina, rectum, and ocular cavity.

46. The method of claim 28, wherein said cavity comprises a mucosal surface.

47. The method of claim 28, wherein said tissue is muscle.

48. The method of claim 47, wherein said tissue is skeletal muscle.

49. The method of claim 28, wherein said administration is intravenous.

10 50. The method of claim 28, wherein said administration is by a route selected from the group consisting of intramuscular, intratracheal, intranasal, transdermal, interdermal, subcutaneous, intraocular, vaginal, rectal, intraperitoneal, intrainestinal and inhalation.

51. The method of any one of claim 28, wherein said administration is mediated
15 by a device selected from the group consisting of a particle accelerator, a pump, an intradermal applicator, a biolistic injector, a pneumatic injector, a sponge depot, a pill and a tablet.

52. The method of claim 28, wherein said administration is mediated by a Biojector®2000.

20 53. A method for providing a mammal a prophylactic or therapeutic treatment associated with a bacterial infection comprising

administering to the mammal an immunogenic composition comprising one or more immunogen-encoding polynucleotides associated with the bacterial infection and an adjuvant composition comprising GAP-DMORIE and a co-lipid, wherein an
25 immunogen is expressed in the mammal in an amount sufficient to generate an immune response to the immunogen.

54. The method of claim 53 wherein the co-lipid is DPyPE.

55. A method for providing a mammal a prophylactic or therapeutic treatment associated with a viral infection comprising

administering to the mammal an immunogenic composition comprising one or more immunogen-encoding polynucleotides associated with the viral infection and an adjuvant composition comprising GAP-DMORIE and a co-lipid, wherein an immunogen is expressed in the mammal in an amount sufficient to generate an immune response to the immunogen.

56. The method of claim 55 wherein the co-lipid is DPyPE.

57. A method for providing a mammal a prophylactic or therapeutic treatment associated with an abnormal growth of a cell population comprising

administering to the mammal an immunogenic composition comprising one or more immunogen-encoding polynucleotides associated with the abnormal growth of the cell population and an adjuvant composition comprising GAP-DMORIE and a co-lipid, wherein an immunogen is expressed in the mammal in an amount sufficient to generate an immune response to the immunogen.

58. The method of claim 57 wherein the abnormal growth of a cell population is associated with cancer.

59. The method of claim 58 wherein the co-lipid is DPyPE.

60. A pharmaceutical kit comprising:

(a) a container holding 1ng to 30 mg of an immunogen-encoding polynucleotide which operably encodes an immunogen within vertebrate cells in vivo; and

(b) an adjuvant composition comprising GAP-DMORIE and a co-lipid, whereby said immunogen is provided in a prophylactically or therapeutically effective amount to treat a vertebrate.

61. The pharmaceutical kit of claim 60, wherein (b) is in the container of (a).

62. The pharmaceutical kit of claim 60, wherein (b) is in the separate container from (a).

63. The pharmaceutical kit of claim 60, further comprising an administration means.

64. The pharmaceutical kit of claim 60, wherein said co-lipid is DPyPE.

65. The pharmaceutical kit of claim 64, wherein said GAP-DMORIE and DPyPE are in molar ratio of about 2:1 to about 1:2.

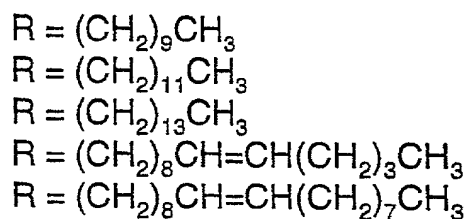
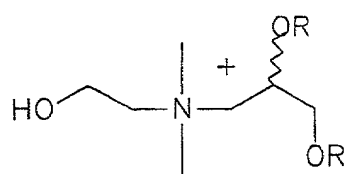
66. The pharmaceutical kit of claim 64, wherein said GAP-DMORIE and DPyPE are in molar ratio of about 1:1.

Figure 1

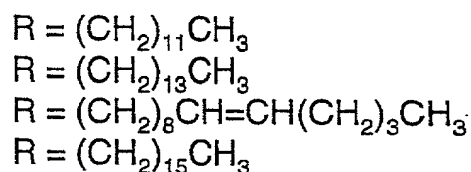
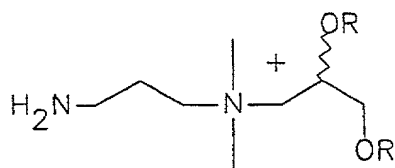
Plasmid	Leader	Gene	Promoter	Enhancer	Intron	Terminator
VR4700	tissue plasminogen activator	influenza A/PR/8/34 nucleoprotein	CMV	CMV	CMV-A	mRBG
VR5900	hen egg lysozyme	hen egg lysozyme	CMV	CMV	CMV-A	mRBG
VR1412	not secreted	E. coli Lac Z	CMV	CMV	CMV-A	BGH
VR1623	mouse immunoglobulin	mouse Ig/humanFc	CMV	CMV	CMV-A	BGH
VR1904	human factor IX	human factor IX	CMV	CMV	CMV-A	BGH

Figure 2

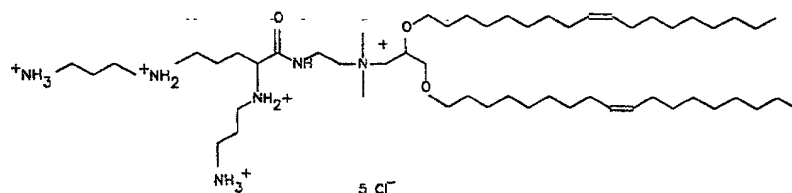
Cytofectin Structures



DDRIE
 DLRIE
 DMRIE
 DMORIE
 DORIE



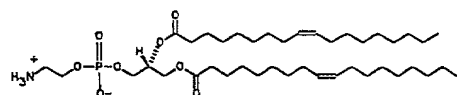
GAP-DLRIE
 GAP-DMRIE
 GAP-DMORIE
 GAP-DPRIE



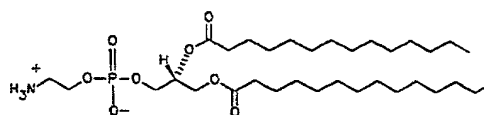
DOSPA

Co-Lipid Structures

DOPE



DMPE



DPyPE

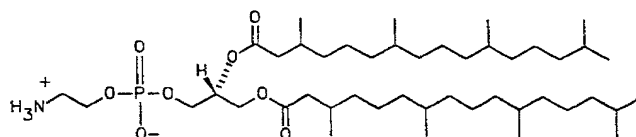


Figure 3

Cytofectin Structural Elements Determine the Level of Antibody Stimulation

Cytofectin Geometric Mean Titer / pDNA Geometric Mean Titer

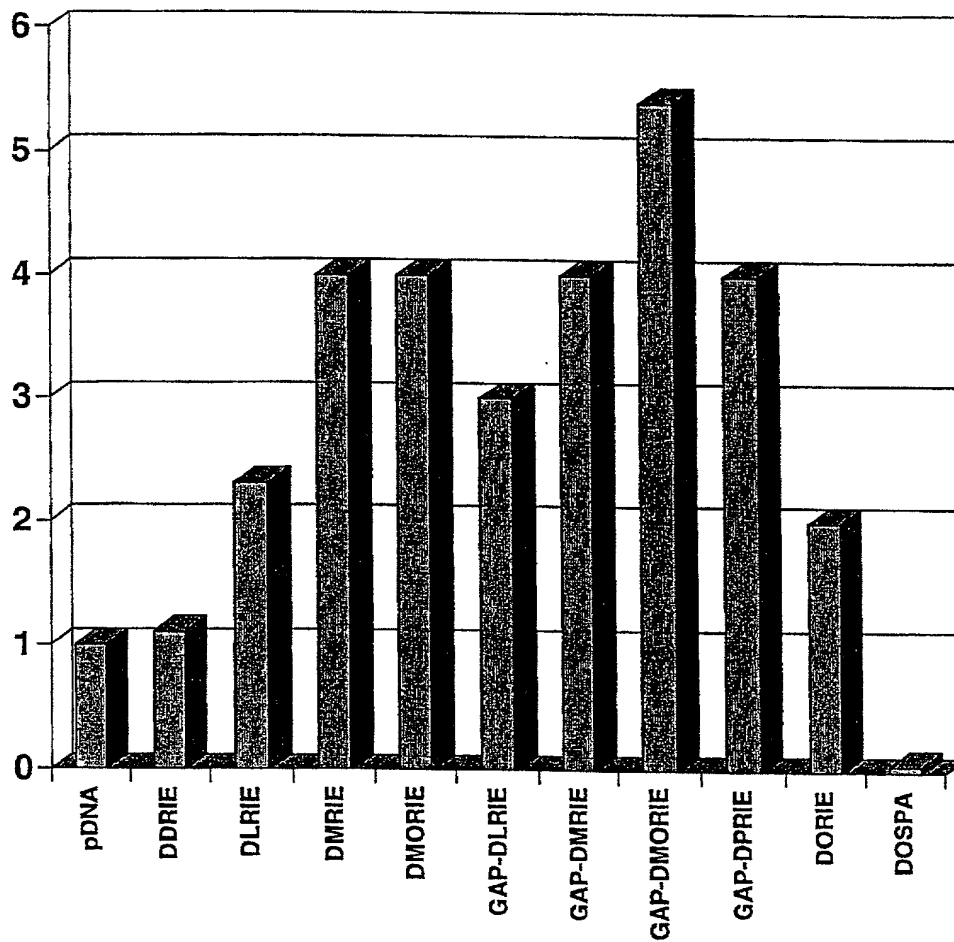
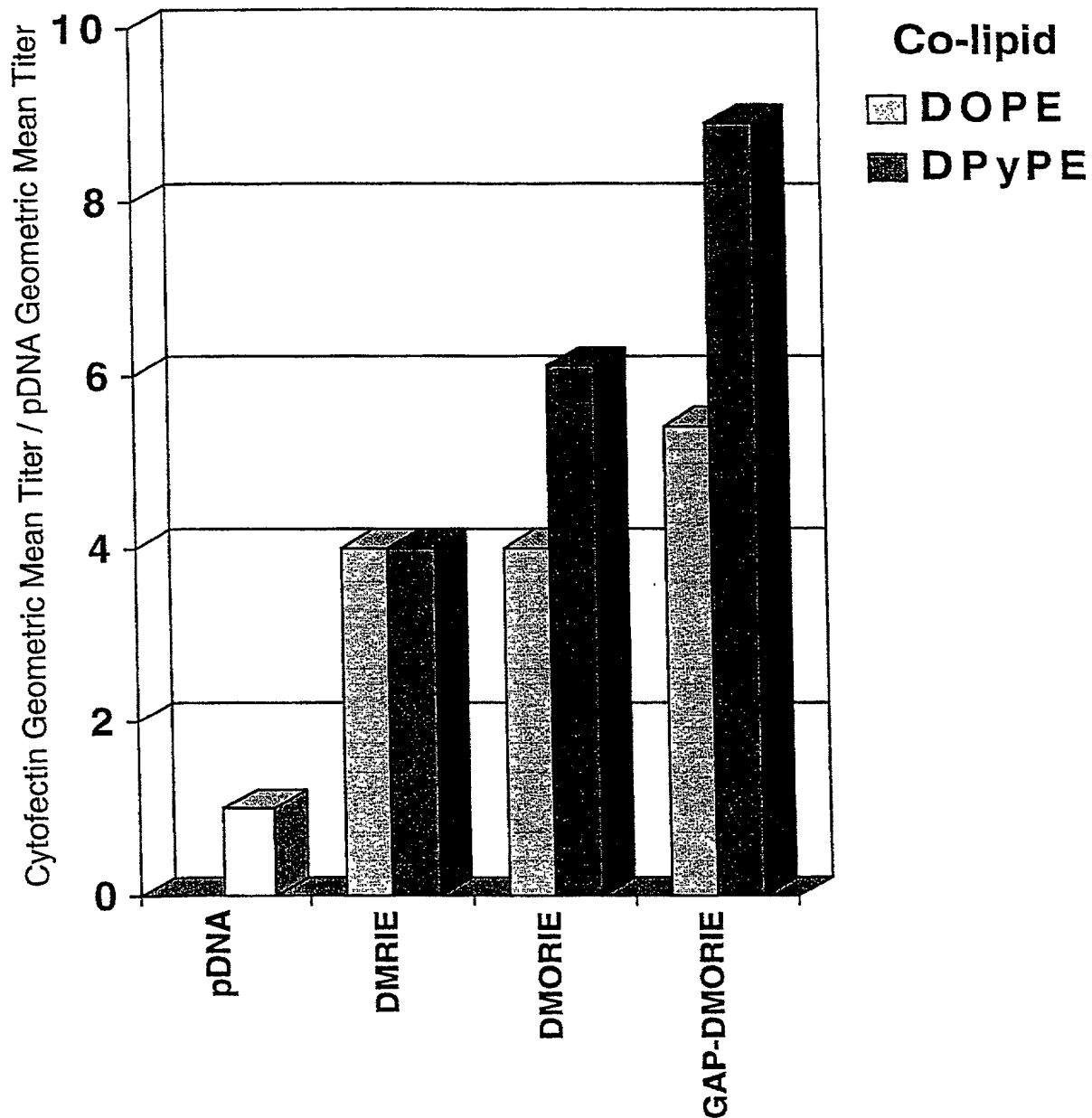
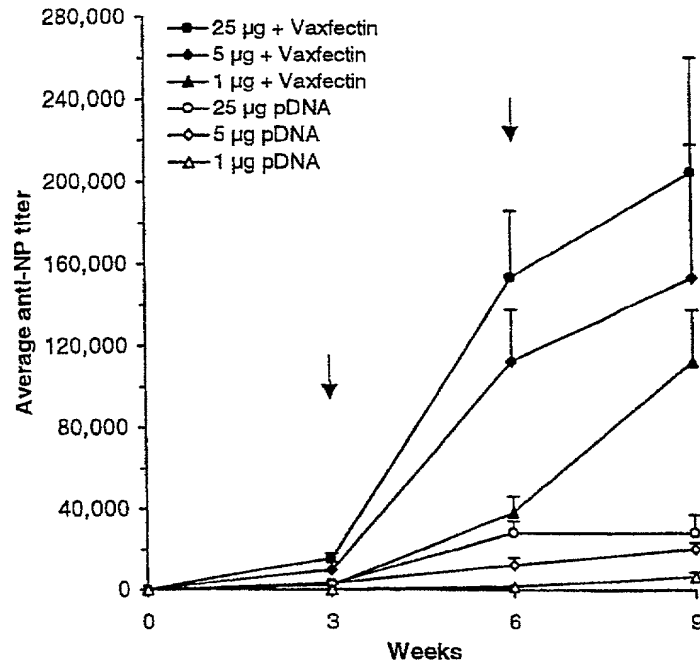


Figure 4

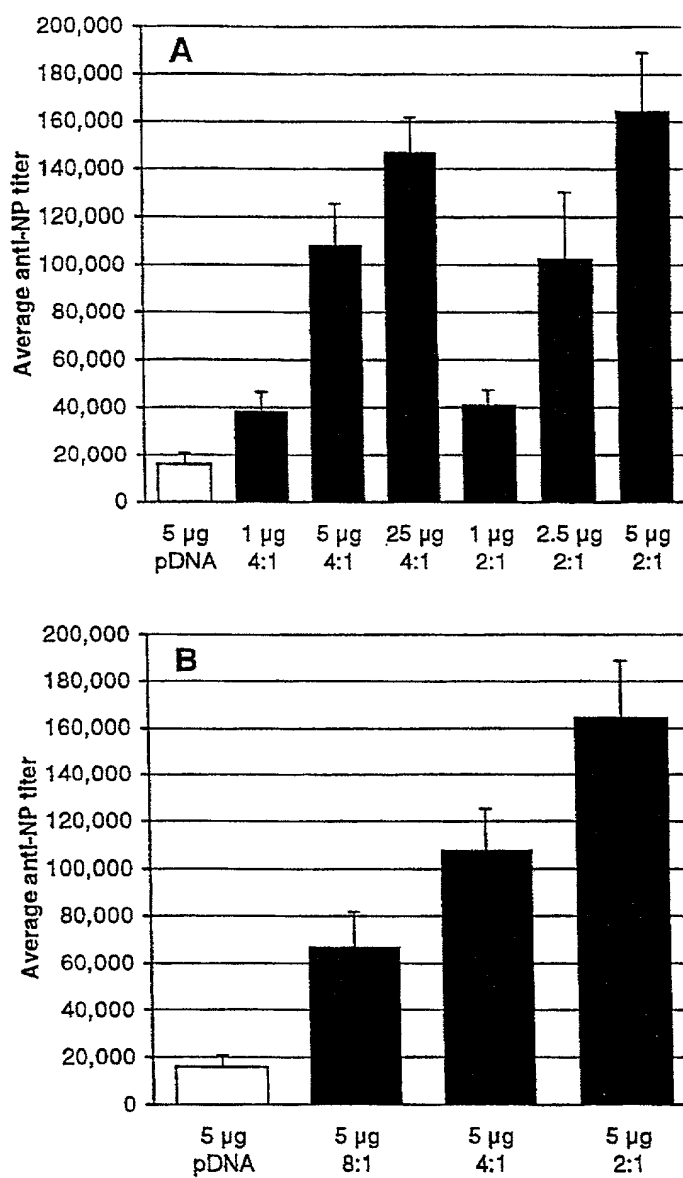
Cytofectins Are Differentially Enhanced By Using DPyPE Instead of DOPE



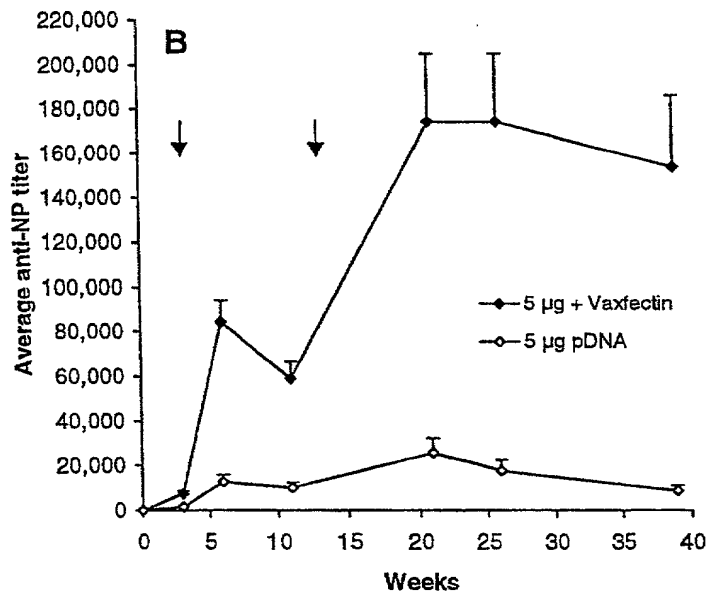
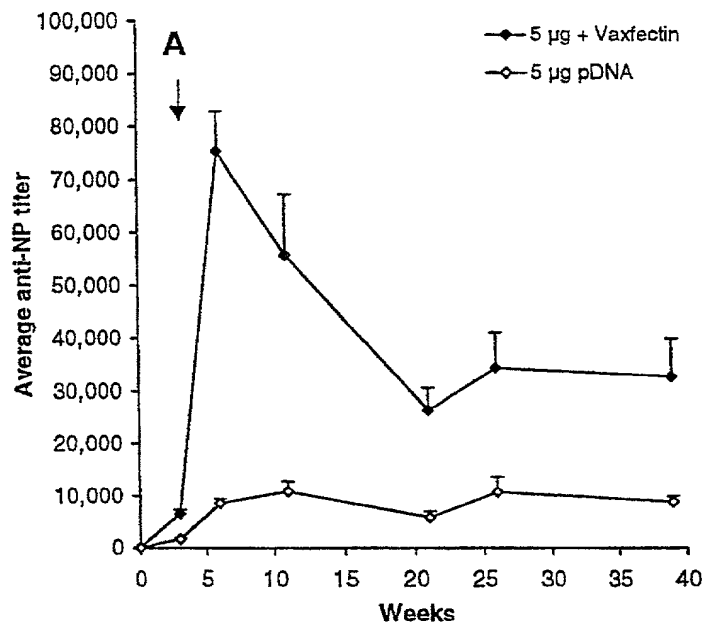
Vaccine, Hartikka et al., Figure 5



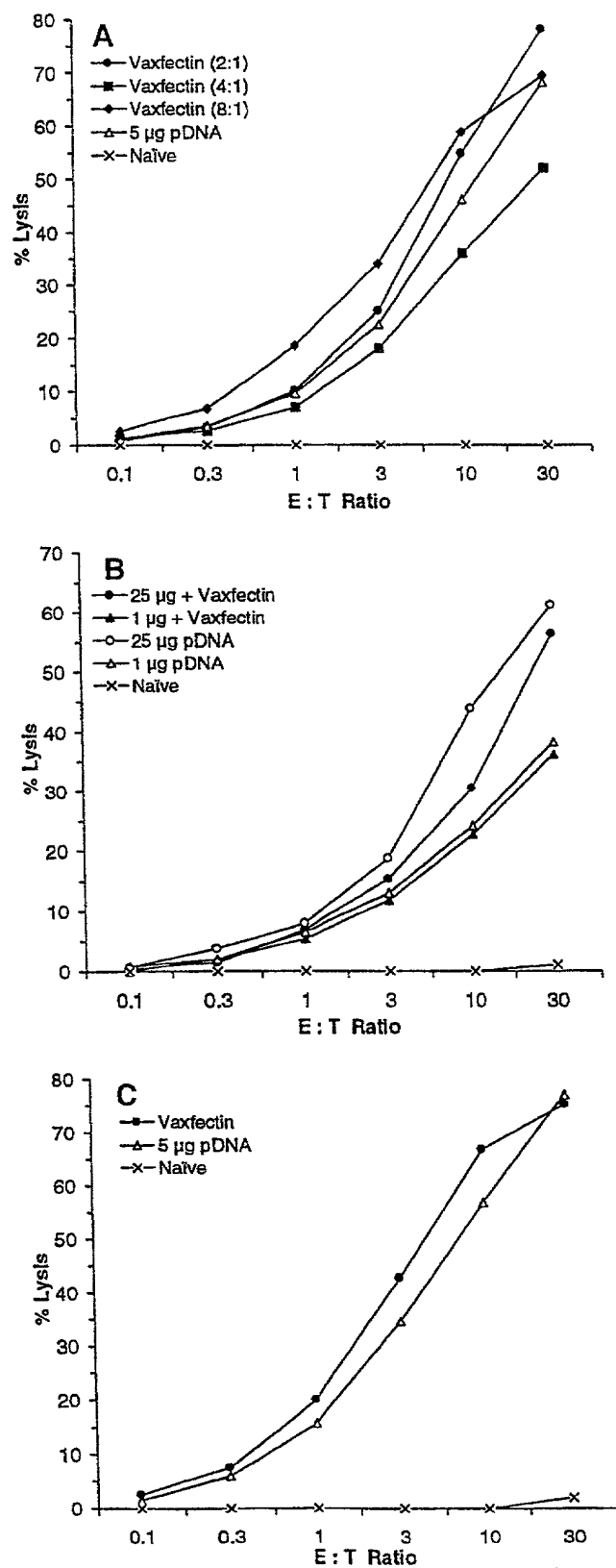
Vaccine, Hartikka et al., Figure 6



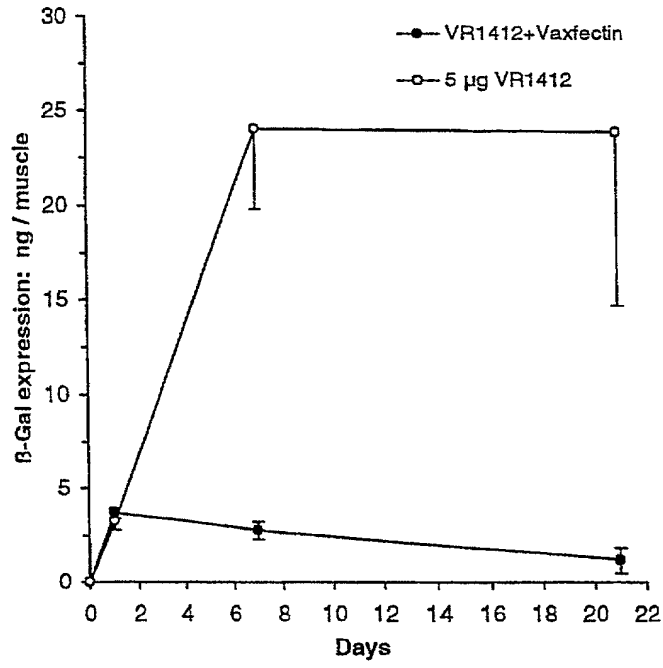
Vaccine, Hartikka et al., Figure 4



Vaccine, Hartikka et al., Figure 8



Vaccine, Hartikka et al., Figure 9



Vaccine, Hartikka et al., Figure 10

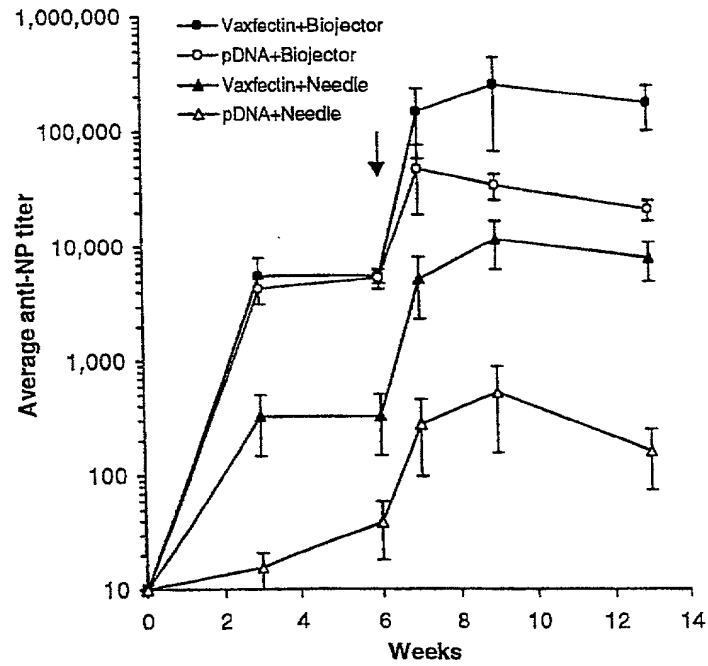


Figure 11

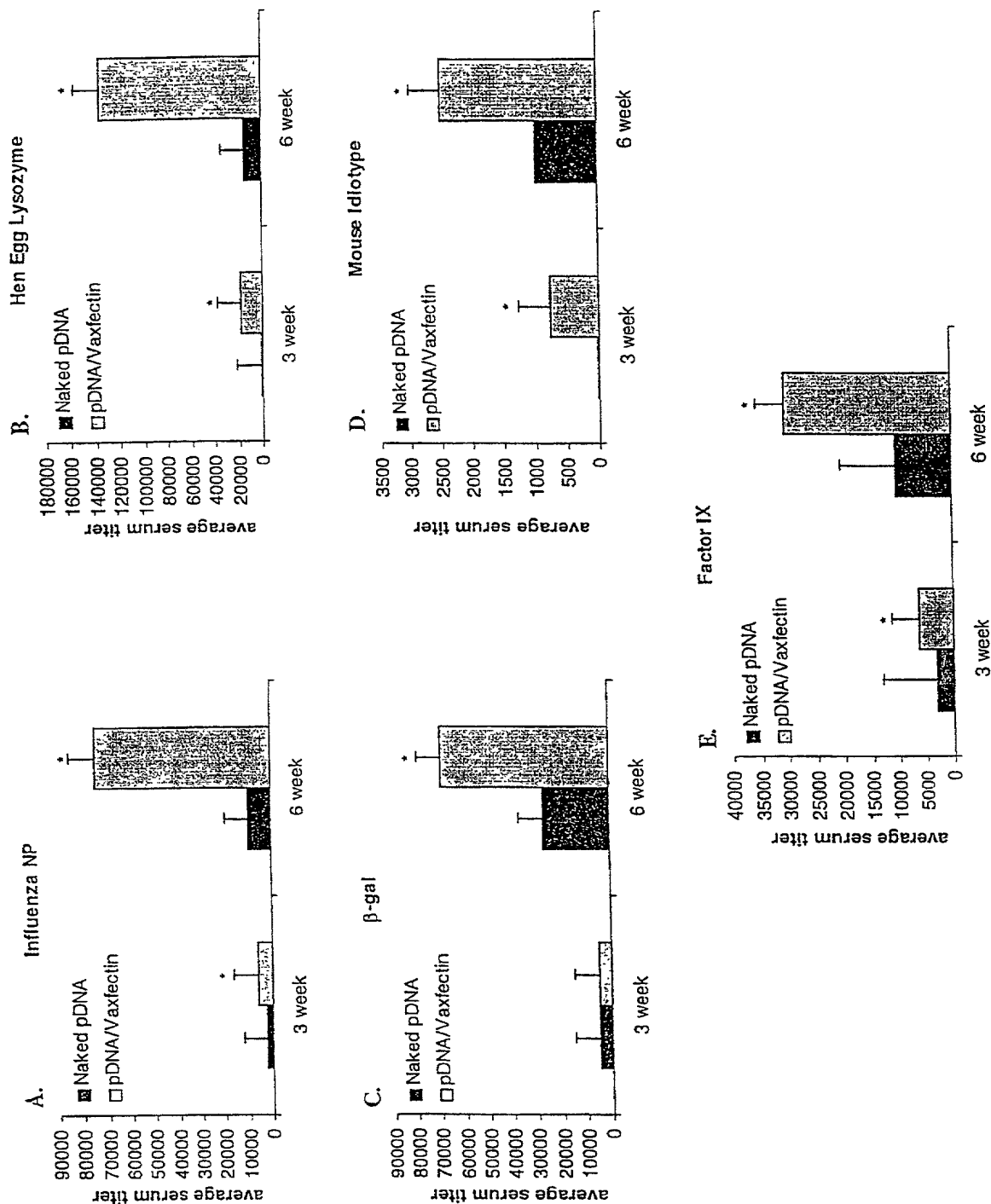


Figure 12

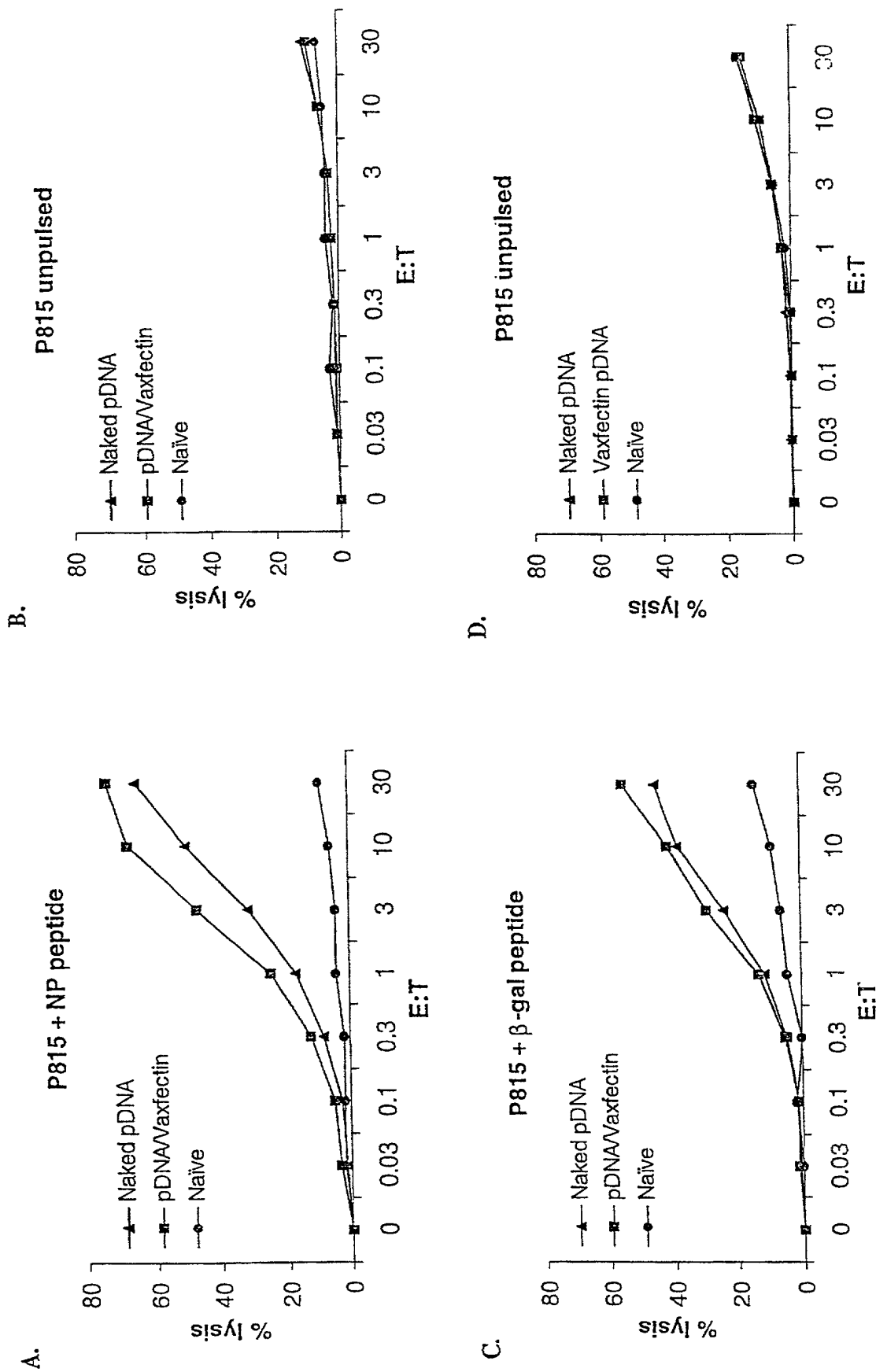
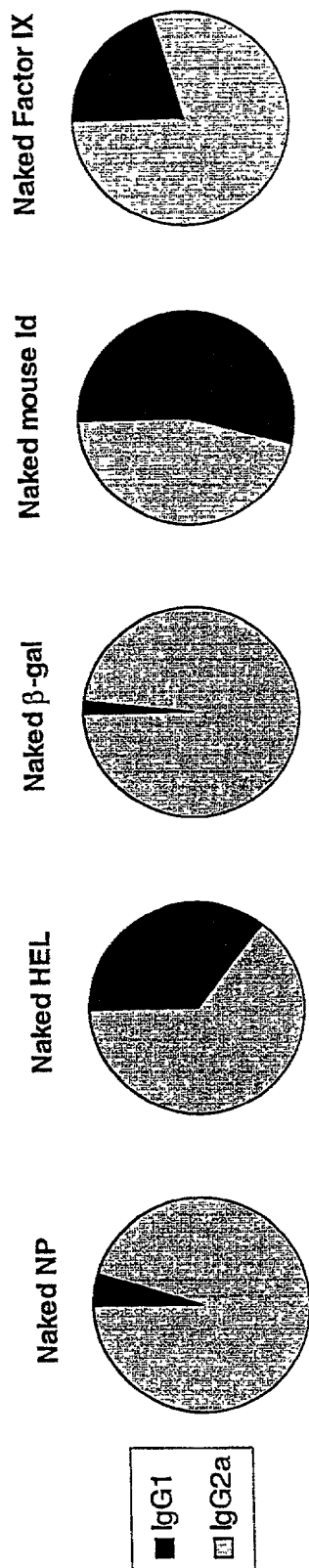


Figure 13

A.



B.

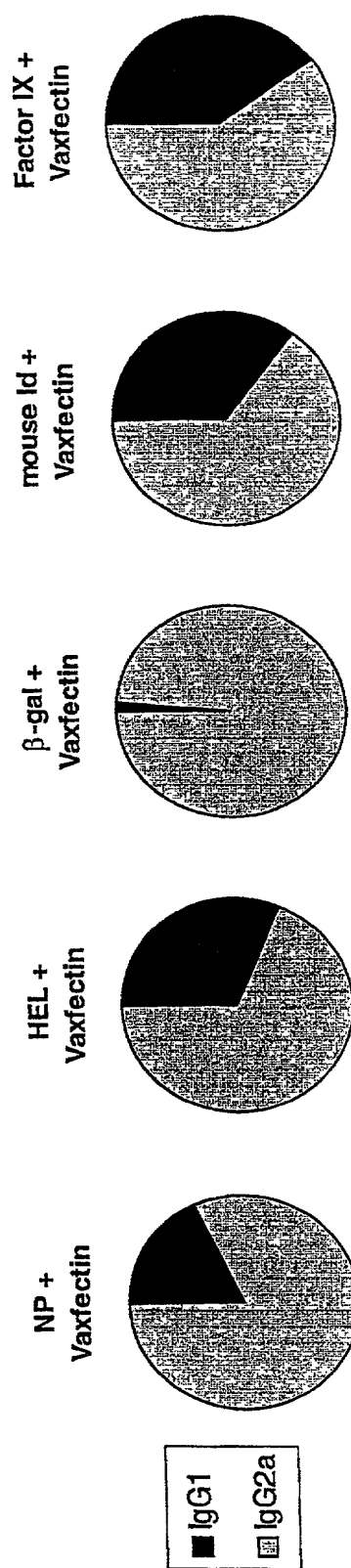
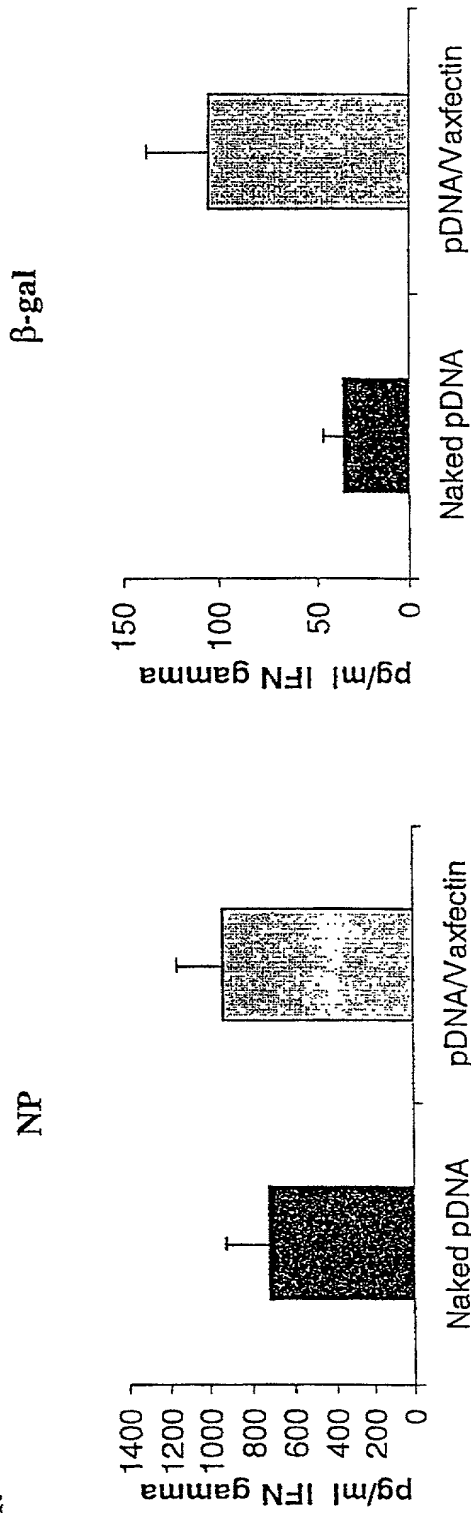
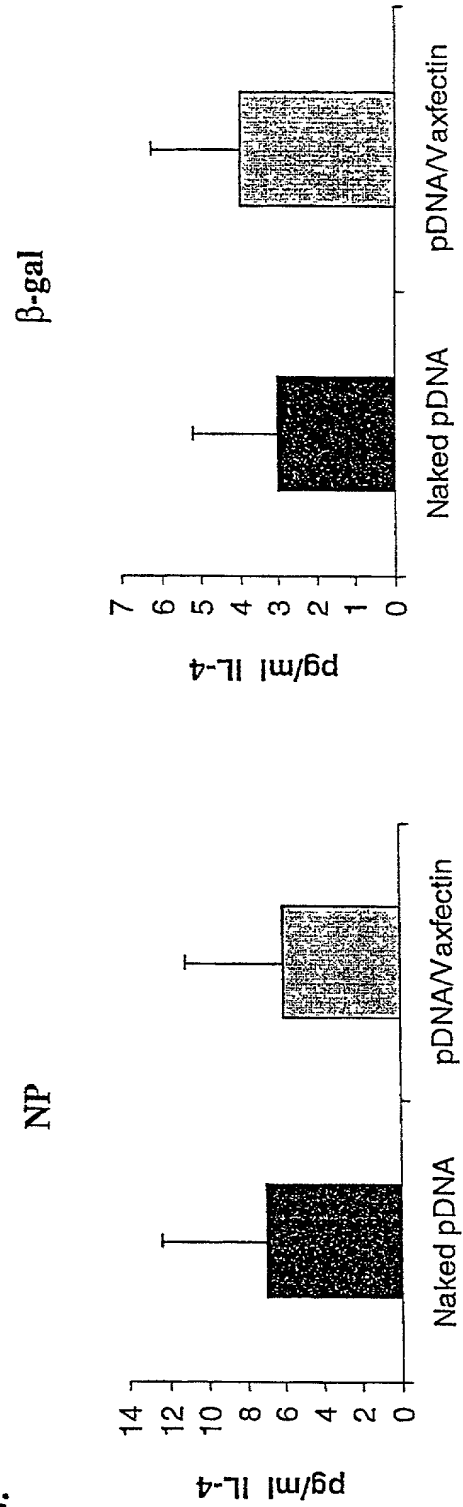


Figure 14

A.



B.



Supplemental Declaration for Patent Application

Docket Number: 1530.0310001/EKS/EJH

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter that is claimed and for which a patent is sought on the invention entitled Adjuvant Compositions and Methods for Enhancing Immune Responses to Polynucleotide-Based Vaccines, the specification of which is attached hereto unless the following box is checked:

- ☒ was filed on March 24, 2000;
as United States Application Number 09/534,943; and
was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information that is material to patentability as defined in 37 C.F.R. § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT international application, which designated at least one country other than the United States listed below, and have also identified below any foreign application for patent or inventor's certificate, or PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)			Priority Claimed	
_____	_____	_____	<input type="checkbox"/> Yes	<input type="checkbox"/> No
(Application No.)	(Country)	(Day/Month/Year Filed)		
_____	_____	_____	<input type="checkbox"/> Yes	<input type="checkbox"/> No
(Application No.)	(Country)	(Day/Month/Year Filed)		

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below.

<u>60/126,340</u>	<u>March 26, 1999</u>
(Application No.)	(Filing Date)
_____	_____
(Application No.)	(Filing Date)

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or under § 365(c) of any PCT international application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information that is material to patentability as defined in 37 C.F.R. § 1.56 that became available between the filing date of the prior application and the national or PCT international filing date of this application.

_____	_____	_____
(Application No.)	(Filing Date)	(Status - patented, pending, abandoned)
_____	_____	_____
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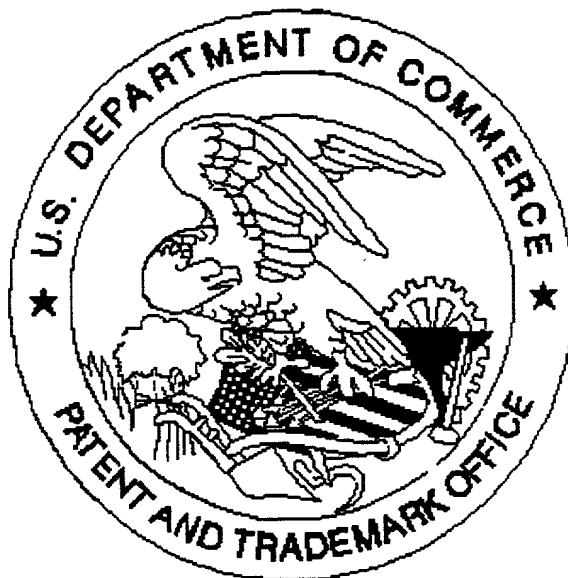
I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or first inventor	Carl J. Wheeler	
Signature of sole or first inventor	<i>Carl J. Wheeler</i>	Date 25 AUGUST 2000
Residence	14101 Arbolitos Drive, Poway, California 92064 CA.	
Citizenship	U.S.A.	
Post Office Address	Same as above	

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(Supply similar information and signature for subsequent joint inventors, if any)

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